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Contributions of the pore-forming toxin *Listeriolysin O* to *Listeria monocytogenes* pathogenesis and immunity

By

Brittney Nhu-Chau Nguyen

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

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University of California, Berkeley

Committee in charge:

Professor Daniel A. Portnoy, Chair
Associate Professor Sarah Stanley
Professor David Raulet

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Abstract

Contributions of the pore-forming toxin *Listeriolysin O* to *Listeria monocytogenes* pathogenesis and immunity

By Brittney Nhu-Chau Nguyen

Doctor of Philosophy in Microbiology

University of California, Berkeley

Professor Daniel A. Portnoy, Chair

Listeriolysin O (LLO) is an essential determinant of *Listeria monocytogenes* pathogenesis that mediates the escape of *L. monocytogenes* from host cell vacuoles, thereby allowing replication in the cytosol without causing appreciable cell death. As a member of the cholesterol-dependent cytolysin (CDC) family of pore-forming toxins, LLO is unique in that it is secreted by a facultative intracellular pathogen, whereas all other CDCs are produced by pathogens that are largely extracellular. Deletion of the gene encoding LLO, *hly*, or replacement of LLO with other CDCs results in strains that are 10,000-fold less virulent during mouse infections. Deletion of LLO also results in a strain that is immunosuppressive in mice. LLO has structural and regulatory features that allow it to function intracellularly without causing cell death, most of which map to a unique N-terminal region of LLO referred to as the PEST-like sequence. Yet, while LLO has unique properties required for its intracellular site of action, extracellular LLO, like other CDCs, affects cells in myriad ways. Because all CDCs form pores in cholesterol-containing membranes that lead to rapid Ca^{2+} influx and K^{+} efflux, they consequently trigger a wide range of host cell responses, including MAPK activation, histone modification, and caspase-1 activation. There is no debate that extracellular LLO, like all other CDCs, can stimulate multiple cellular activities, but the primary question we wish to address is whether LLO secreted in the cytosol has an impact on pathogenesis.

To address whether LLO secreted in the cytosol impacts the pathogenesis of *L. monocytogenes* we engineered a strain, referred to as *hly*^{fl}, that deletes *hly* after escape from phagocytic vacuoles. Using *hly*^{fl}, we determined that LLO secreted in the cytosol causes cytotoxicity that impairs the growth of *L. monocytogenes* in macrophages. However, this strain was less virulent in mice than WT *L. monocytogenes* because it had a defect in cell-to-cell spread. Furthermore, the strain was not as effective at inducing protective immunity in mice as a strain that was defective in cell-to-cell spread due to a defect in actin-based motility. We hypothesized that *hly*^{fl} was not as effective at inducing protective immunity because, like LLO-deficient strains, it induced IL-10. Administration of anti-IL-10 receptor blocking antibody improved the protective capacity of vaccination with *hly*^{fl}, indicating that bacterial localization in primary and secondary vacuoles leads to the induction of IL-10, which is immunosuppressive.

To determine the bacterial components that lead to the induction of IL-10 by bacteria that cannot escape phagocytic vacuoles, we performed a genetic screen of transposon mutants for their ability to induce enhanced or diminished IL-10 from infected bone marrow-derived macrophages. We identified bacterial lipoproteins, which are recognized by TLR2, as the primary signal for IL-10 induction in macrophages. However, bacterial mutants that had increased lysis induced enhanced IL-10, which was dependent on signaling of endosomal TLRs. In mice, IL-10 induction and immune suppression was primarily mediated by endosomal TLRs. In summary, though LLO may cause some cytotoxicity it is absolutely required for escape from primary and secondary vacuoles. LLO-deficient mutants are avirulent, and they also lyse in vacuoles and induce expression of IL-10, which is immunosuppressive.

Dedication

In memory of Dr. Jay Vavra,
who inspired me to become a good scientist,
and showed me how to be a good person.

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To my family, thank you. My grandparents believed that there is nothing more important than education. My grandparents and parents worked tirelessly so that I never had to worry about paying for school, and could instead focus on my education. Because of them, I have been able to commit myself to intellectual pursuits.

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Chapter 1
Listeriolysin O: Swiss Army knife or phagosome-specific lysin?

1.1 Introduction to Listeria monocytogenes

L. monocytogenes is a Gram-positive bacterium that is ubiquitous in the environment but can become a facultative intracellular pathogen upon ingestion of *Listeria*-contaminated foods (1). *L. monocytogenes* primarily affects immunocompromised individuals, including pregnant women and the elderly. Furthermore, infection with *L. monocytogenes* leads to the induction of a robust adaptive immune response that is protective against subsequent infections. As *L. monocytogenes* is a genetically-tractable organism that is easily manipulated in laboratory environments, it has become a model for intracellular bacterial pathogens. Additionally, many are trying to take advantage of the immune responses induced by *L. monocytogenes* to develop live *Listeria*-based anti-cancer vaccines.

specifically replicates in the cytosol of host cells. In order to reach the host cell cytosol, *L. monocytogenes* must first escape from the phagocytic entry vacuole, which requires the secreted cholesterol-dependent cytolysin Listeriolysin O (LLO) (2,3). In the cytosol, *L. monocytogenes* replicates and produces an actin-nucleation factor (ActA) to move intracellularly and form protrusions that are engulfed by neighboring cells and resolved into double-membraned vacuoles. Again, LLO is required for escape from these secondary vacuoles (4,5).

1.2 Introduction to Cholesterol-Dependent Cytolysins

Cholesterol-dependent cytolysins (CDCs) represent the largest family of pore-forming toxins (PFTs) and the subset of PFTs that form the largest pores (6). To date, more than 50 CDCs have been identified in *Firmicutes, Actinobacteria*, and most recently in *Proteobacteria* (7,8). With the exception of those produced by *Proteobacteria,* CDCs are produced by primary and opportunistic Gram-positive pathogens, and many have important roles in pathogenesis. Among CDCs that have demonstrated contributions to pathogenesis are perfringolysin (PFO) of *Clostridium perfringens,* pneumolysin (PLY) of *Streptococcus pneumoniae,* streptolysin O (SLO) of *Streptococcus pyogenes,* anthrolysin (ALO) of *Bacillus anthracis,* and listeriolysin O (LLO) of *Listeria monocytogenes.* LLO is distinct in that it is the only CDC produced by an intracellular pathogen and has specialized features that make it suitable for its intracellular localization.

The hallmarks of CDCs are their requirement of membrane cholesterol for pore-forming activity, and their extremely large pores—which can be 30 - 40 nm in diameter (9,10). CDCs also contain a singular conserved cysteine that makes them highly sensitive to oxidation; CDCs were once classified as ‘thiol-activated (oxygen-sensitive) cytolysins’ because they required reducing agents for maximal activity (11,12).

CDCs are secreted via a Sec-dependent pathway as monomers 50-70 kDa in mass and consist of four distinct domains. Secreted monomers bind to cell membranes and oligomerize into arc and ring prepore assemblies, which may contain up to 50 subunits. Following membrane binding, α-helical regions in domain 3 of each monomer refold into two β-hairpins that insert into the membrane and form a β-barrel pore (10,13,14). Domain 4 contains the signature undecapeptide sequence (ECTGLAWEWWR) that is the most highly conserved region in the primary CDC sequence and is required for coupling of cholesterol binding to domain 3 rearrangement (15). The cholesterol recognition/binding motif, which consists of a threonine-leucine pair, is also located in domain 4 (16). Both incomplete ring oligomers (arcs or slits) and complete rings perforate cell membranes, though pores formed by arcs are considerably smaller and may only function as ion channels, while rings allow the translocation of fully folded

proteins (17). There is evidence that CDCs translocate proteins *in vivo*, thereby acting analogously to type III secretion systems or the β-subunits of other secreted toxins (18).

Although cholesterol is required for CDC activity and is generally considered the CDC receptor, a number of CDCs use human CD59 as a receptor and consequently have increased specificity for human cell membranes. However, these CDCs still require cholesterol for pore formation. These include intermedilysin (ILY) of *Streptococcus intermedius*, vaginolysin (VLY) of *Gardnerella vaginalis*, and lectinolysin (LLY) of *Streptococcus mitis.* The use of CD59 as a receptor may be attributed to a proline residue in place of a tryptophan in the undecapeptide (19). Additionally, CDCs also have conserved lectin-binding properties (20). Using glycan array analysis, it was shown that PLY and SLO had affinities for different glycan structures and that binding these glycans altered the hemolytic activity of these toxins. Like cholesterol, the functional domain responsible for glycan binding is domain 4. While glycan binding has not yet been reported for LLO, many of the modeled carbohydrate binding sites within domain 4 are conserved between LLO and CDCs from extracellular pathogens. Future experiments should investigate the roles of glycosylation with respect to cellular tropism and pathogen lifestyle.

Although the structure and mechanism of pore formation of CDCs are largely conserved, several CDCs have variations in their structure that contribute to changes in function. SLO has 60 amino acids at its N-terminus that mediate specific translocation of NAD+ glycohydrolase (SPN) into keratinocytes (18). Translocation of SPN induces cell death, following depletion of cellular NAD+, and significantly increases the virulence of *S. pyogenes* (21,22). PLY lacks a signal peptide and may be released by cell lysis or by another export mechanism (23). PLY also localizes to the cell wall, and its cell wall localization is dependent on SecY2A2, an accessory Sec system (24). LLO has a 26-amino acid addition (known as the PEST-like sequence) near its N-terminus that reduces intracellular toxicity, allowing *L. monocytogenes* to escape phagocytic vacuoles and survive intracellularly (25). The role of the LLO PEST-like sequence in pathogenesis will be discussed in depth below.

1.3 Cellular Responses to CDC-Mediated Pore Formation

CDCs can induce a wide range of effects in cells, including activation of membrane damage responses and alteration of immune cell function. Among other things, CDCs can activate MAPKs, caspase-1, and TLR4, modulate SUMOylation, induce mitochondrial fragmentation, and enhance bacterial internalization (26–28). These responses are usually common to membrane insult by a range of PFTs and are often the direct result of Ca^{2+} influx and/or K^+ efflux. As a result of these numerous and diverse effects, LLO has been called the ‘Swiss-army knife of *Listeria’* (Hamon, Ribet, Stavru, & Cossart, 2012; Osborne & Brumell, 2017). However, the role of these cellular responses in pathogenesis is not clear.

For more details on cellular responses to pore formation, refer to the following references: Cajnko et al., 2014, Cassidy & O’Riordan, 2013, Seveau, 2014, and Gonzalez, Bischofberger, Pernot, van der Goot, & Frêche, 2008.

1.3.1 Mitogen-activated Protein Kinase (MAPK) activation

MAPKs are involved in the initiation of signaling cascades that activate cellular responses to many stimuli. Cell membrane damage by PFTs causes the rapid efflux of intracellular K^+, and activation of the MAP kinases p38, extracellular signal-regulated kinase (ERK), Jun N-terminal kinase (JNK), mitogen- and stress-activated kinase 1 and 2 (MSK1/2), and cAMP response-element binding protein (CREB). Activation of p38 and ERK are required for recovery of

intracellular K^+ levels following treatment of cells with sublytic concentrations of LLO and aerolysin, a non-CDC PFT that forms 2 nm pores (Cabezas et al., 2017; Gonzalez et al., 2011). In *Caenorhabditis elegans*, p38 and JNK MAPK pathways, and importantly one downstream target, activator protein 1 (AP-1), provide protection against PFT toxicity (34). Thus, the restoration of ion homeostasis is one effect of MAPK activation in response to membrane perforation by PFTs.

1.3.2 Histone Modification

Histone modification has been observed in response to multiple pathogens and their CDCs. *L. monocytogenes* infection causes phosphorylation or dephosphorylation of Ser10 in histone H3 and acetylation or deacetylation of histone H4, depending on the experimental conditions (Hamon et al., 2007; Schmeck et al., 2005). In human umbilical vein endothelial cells, *L. monocytogenes* infection caused phosphorylation of Ser10 in histone H3, leading to increased expression of numerous cytokines in a p38 MAPK-dependent manner (36). Conversely, in human cervical epithelial cells (HeLa cells), *L. monocytogenes* infection or LLO alone caused dephosphorylation of Ser10 in histone H3, deacetylation of histone H4, and transcriptional repression of *cxcl2*, a cytokine involved in inflammation and neutrophil chemotaxis (Hamon et al., 2007). Treatment of HeLa cells with aerolysin, PFO, and PLY also results in dephosphorylation of Ser10 in histone H3, and dephosphorylation is dependent on K^+ efflux (Hamon & Cossart, 2011). Part of the *Pseudomonas aeruginosa* Type III secretion system, the PopB-PopD translocon, can form 4 nm pores on cell membranes that also result in K^+ efflux-dependent dephosphorylation of Ser10 in histone H3 in HeLa cells (38). Thus, K^+ efflux resulting from membrane pore formation may have the ability to alter transcriptional profiles in ways that affect inflammation, although a role *in vivo* has not been demonstrated.

1.3.3 Alteration of Mitochondrial Dynamics

Treatment of HeLa cells with a sublytic concentration of recombinant LLO caused mitochondrial fragmentation, defined by breakage of the mitochondrial network into visually punctate structures. Mitochondria fragmentation occurred quickly after LLO treatment, and was transient; *L. monocytogenes*-infected cells completely recovered their normal mitochondrial network phenotype overnight, and did not become apoptotic. Mitochondrial fragmentation was dependent on Ca^{2+} influx, as cells incubated in Ca^{2+}-free media did not undergo mitochondrial fragmentation upon LLO addition (39). In addition to causing histone modifications, infection of HeLa cells with *P. aeruginosa* also caused mitochondrial fragmentation, and was dependent on the expression of PopB and PopD (38).

1.3.4 SUMOylation

SUMOylation is a eukaryotic post-translational modification, similar to ubiquitylation, in which small ubiquitin-like modifier (SUMO) is covalently attached to proteins. Most commonly, SUMOylation of transcriptional regulators leads to transcriptional repression (40). Treatment of HeLa cells with LLO, PFO, PLY, and suilysin (SLY)—a CDC produced by *Streptococcus suis*—resulted in degradation of Ubc9, an E2 SUMO enzyme, and a reduction in SUMO-conjugated proteins (Li, Lam, Lai, & Au, 2017; Ribet et al., 2010). Interestingly, the patterns of SUMOylated proteins were different for each CDC. Blockage of K^+ efflux prevented LLO-induced degradation of Ubc9, but enhanced Ubc9 degradation induced by PFO, PLY, and SLY, suggesting CDCs have different mechanisms for inducing Ubc9 degradation (Li et al., 2017).

Overexpression of SUMO 1 and SUMO 2 in HeLa cells limited infection of *L. monocytogenes* 2-fold (41). Based on the observation that mice deficient for promyelocytic leukemia protein (PML), a known target of SUMO, had a defect in controlling *L. monocytogenes*, a recent study investigated the relationship between LLO, PML and SUMO during infection (43,44). The authors reported that treatment with LLO, PFO, and PLY caused deSUMOylation of PML in Chinese hamster ovary (CHO) cells, and that gene expression of many cytokines was reduced in *pml*$^{-/-}$ mouse embryonic fibroblasts (MEFs) compared to *pml*$^{+/+}$ MEFs after *L. monocytogenes* infection, though the altered gene expression was not dependent on LLO (44). A better understanding of how SUMOylation affects pathogenesis can be acquired by performing studies in mice with conditional deletions of *Ubc9* (45–47).

1.3.5 Caspase-1 activation

One of the hallmarks of the innate immune system is that activation of host pattern recognition receptors by conserved microbial products, known as pathogen-associated molecular patterns (PAMPs), and aberrant structure or localization of host molecules that result during disease, often referred to as damage-associated molecular patterns (DAMPs), leads to the initiation of immune responses (48–52). An example of a DAMP is extracellular ATP, which binds and activates P2X7, an ATP-gated ion channel. Binding of ATP to P2X7 results in K^+ efflux and K^+ efflux-dependent NLRP3 inflammasome activation (53–55). Caspase-1 cleaves pro-interleukin 1 beta (IL-1β) into active IL-1β downstream of NLRP3 activation, which can occur spontaneously in low concentrations of K^+ (55,56). Many PFTs activate the NLRP3 inflammasome, including the pore-forming component of adenylate cyclase toxin (CyaA) produced by *Bordetella pertussis*, aerolysin, SLO, LLO and tetanolysin, a CDC produced by *Clostridium tetani* (53,54,57–61). Roles for the inflammasome in the pathogenesis of *B. pertussis*, *S. pneumoniae*, and *L. monocytogenes* have been reported, although in the case of *L. monocytogenes*, excess inflammasome activation reduces virulence (61–68).

1.4 Contributions of CDCs to Pathogenesis of Extracellular Pathogens

Many CDCs have clear roles in pathogenesis that are often related to the recruitment of immune cells or disruption of tissue barriers. Though all CDCs function by forming pores on host membranes, there are some differences in how they contribute to pathogenesis. Differences in how CDCs function *in vivo* are likely due to differences in infection sites and the simultaneous effects of other bacterial factors.

1.4.1 PFO

Upon introduction of *C. perfringens* into soft tissue, often by a traumatic injury, *C. perfringens* can cause clostridial myonecrosis, a necrotic infection of muscle that is largely devoid of infiltrating leukocytes (69,70). Although *C. perfringens* produces many toxins, PFO (also called θ–toxin) is critical for severe pathology. PFO acts synergistically with α-toxin, a phospholipase produced by *C. perfringens*, in the development of clostridial myonecrosis (71). α-toxin causes most of the damage to the muscle tissue, while PFO contributes to pathology by inducing leukocyte aggregation in the vasculature—thereby preventing infiltration of leukocytes into the site of infection (72). Deletion of PFO, but not α-toxin, in a mouse muscle model resulted in an almost complete reduction in severe leukocyte accumulation, which was likely caused by the upregulation of leukocyte and endothelial cell adhesion factors (Bryant & Stevens, 1996; Ellemor et al., 1999; Verherstraeten et al., 2015). Treatment of mice with a PFO-

neutralizing antibody prior to infection with a lethal dose of intramuscular *C. perfringens* significantly reduced mortality (Bryant et al., 1993). Additionally, PFO is required for persistence of *C. perfringens* in a low-dose mouse femoral muscle infection model (76). Disruption of leukocyte migration and subsequent inflammation induced by PFO contributes to the disease progression of clostridial myonecrosis.

1.4.2 PLY

S. pneumoniae is the causative agent for a number of diseases, including pneumonia, otitis media, meningitis, and sepsis (77). PLY has a significant role in the pathogenesis of pneumococcal pneumonia and sepsis (78,79). In an intranasal mouse infection model of pneumonia and septicemia, mice infected with PLY-negative bacteria did not develop severe disease, whereas mice infected with *S. pneumoniae* expressing PLY were moribund within 48 hours. Mice infected with the PLY-negative mutant had 4-logs less bacteria in both the lungs and blood (80). In addition, mice treated with a PLY-neutralizing antibody prior to infection with a lethal dose of *S. pneumoniae* administered intranasally or intraperitoneally had significantly increased survival (81,82). PLY reduced ciliary beating and caused reduced or disorganized cilia on the epithelial cells of human adenoid organ cultures. This result correlated with increased numbers of *S. pneumoniae* adhering to the organ cultures, mostly on damaged cells near disrupted tight junctions (83). Therefore, PLY may contribute to dissemination of *S. pneumoniae* during infection of the lungs by disrupting mucociliary elevator-mediated bacterial clearance and allowing the bacteria to invade deeper tissues through disrupted tight junctions.

PLY-induced IL-1β secretion provides partial protection to the host during *S. pneumoniae* infections. IL-1β secretion in response to PLY is caspase-1, NLRP3, and ASC-dependent in many cell types (67,68,84). IL-1β$^{-/-}$, caspase-1/11$^{-/-}$, ASC$^{-/-}$ and NLRP3$^{-/-}$ mice had 1-log increased bacterial burdens compared to WT mice in a *S. pneumoniae* keratitis model (68). In a mouse lung infection model, *S. pneumoniae* infection caused increased lung permeability, which was exacerbated in mice lacking NLRP3 (66). IL-1β contributed to the activation of γδT cells, and their production of IL-17A. IL-17A-deficient, TCRδ-deficient, and neutrophil-depleted mice had significantly reduced survival compared to WT mice (67). Thus, inflammasome activation by PLY leads to the generation of the Th17 response, which provides some protection against *S. pneumoniae* infection.

1.4.3 ALO

Bacillus anthracis is capable of causing severe disease in humans after inhalation, ingestion, or cutaneous introduction of bacterial spores. In all cases, the disease begins as a localized infection that can quickly lead to sepsis (85). ALO plays a significant role in pathogenesis (86). Administration of 100 μg of ALO-neutralizing antibody to mice infected with a lethal intravenous dose of *B. anthracis* significantly increased survival (87). ALO is required for disruption of tight junctions and the gut epithelial barrier, and for the apical to basolateral translocation of *B. anthracis* across C2BBE monolayers, suggesting that the function of ALO is to facilitate the early establishment and penetration of *B. anthracis* into the gut epithelium (88). Though significant, the role of ALO in systemic infection is not well characterized.

1.5 Varying Roles for CDCs in Pathogenesis

The role of CDCs in the pathogenesis of extracellular pathogens often involves damaging, but not necessarily killing, cells in and around the site of infection. CDCs cause cell remodeling,

such as ciliary rearrangement, which may promote bacterial adhesion; they can disrupt tight junctions, facilitating bacterial translocation through epithelial and endothelial barriers and thus facilitating dissemination; and they can alter the expression of adherence factors, which can lead to recruitment of phagocytes and inflammation, or prevention of immune cell infiltration. Though many of these functions do not result in cell death, host cell lysis can also be a survival strategy for extracellular pathogens. Indeed, both *C. perfringens* and *B. anthracis* use their CDC to lyse host cells subsequent to phagocytosis, thereby releasing the bacteria back into the extracellular space and promoting bacterial growth (76,89). Cell lysis may also provide extracellular pathogens with nutrients. These CDC-mediated effects are well suited to the needs of extracellular pathogens.

1.5.1 LLO

L. monocytogenes is a facultative intracellular pathogen that, in humans, primarily causes self-resolving gastrointestinal infections. In immunocompromised individuals, *L. monocytogenes* can cause fatal systemic infections and, in pregnant women, placental infections that lead to pregnancy loss and systemic disease that results in death to the neonate (90). LLO is required for virulence in most if not all *L. monocytogenes* animal disease models, including acute systemic infection in mice, neonatal mice, pregnant mice and pregnant guinea pigs (65,91–96). The requirement for LLO in virulence can be recapitulated in tissue culture where it is required for *L. monocytogenes* to escape from a phagosomal membrane (97). Mutants lacking LLO are unable to escape from the phagosome and consequently unable to grow intracellularly. LLO-negative mutants are 5-logs less virulent in mice and cause immunosuppression via induction of the cytokine IL-10. Strikingly, replacement of LLO with other CDCs results in strains that can escape from a phagosome but then kill the infected host cell, thereby eliminating the intracellular replicative niche (2,98–100).

The optimal pH for LLO activity is 5.5, while extracellular CDCs such as PFO and SLO have similar activities at pH 5.5 and pH 7, suggesting that LLO has adapted to the specific setting of the acidified phagosome (98,101). An early study into the molecular basis of this low optimal pH found that amino acid L461 was the main determinant, and that this leucine is not conserved in extracellular pathogens (102). Nonsynonymous mutations of L461 affect LLO activity and cytotoxicity. Mutants with a threonine substitution, the residue common in extracellular pathogen CDCs, were 100-fold less virulent in mice due to their increased cytotoxicity. The pH insensitivity of L461T may be caused by an increase in the rate of oligomerization. Later it was reported that LLO is denatured at neutral pH at temperatures greater than 30°C, and that this was caused by charged amino acids within the transmembrane helices of domain 3 that act as a pH sensor (103). Thus, while LLO is maximally active in acidified phagosomes, in the host cell cytosol its activity is partially reduced and it has the potential to denature. This mechanism is not solely responsible for limiting the activity of LLO to the phagosome, but it does contribute to reducing LLO-mediated cytotoxicity and preserving the replicative niche.

1.5.2 The LLO PEST-like Sequence

The most distinctive and single largest contributing feature of LLO for the *L. monocytogenes*-specific lifestyle is a PEST-like sequence at the amino terminus of the protein (100,104). PEST-like sequences were originally described in eukaryotic proteins with short half-lives and were thought to mediate those short half-lives, but it is now appreciated that they often

include another domain known as the polyproline type II (PPII) helix that mediates protein-protein interactions (105–107). Structural and *in vitro* analyses have indicated that residues in PPII helix region play a role in oligomerization through intermolecular contacts (107). Deletion of 26 amino acids of LLO that include the PEST-like sequence has a minor effect on hemolytic activity; however, the bacteria are extremely cytotoxic in tissue culture and 10,000-fold less virulent in mice (100).

Intracellular LLO exists in multiple forms, including 58kDa and 55kDa molecular weight species. The lighter species is absent during infection with the PEST-deletion mutant or mutants deficient in actin-based motility, suggesting the PEST-like sequence contributes to subcellular compartmentalization or processing of LLO (108). Additionally, independently of the PEST-like sequence, LLO is ubiquitylated and accumulates as a ladder of higher molecular weight species in the presence of proteasome inhibitors. LLO has an N-terminal lysine that serves as a destabilizing signal for the N-end rule pathway, which involves ubiquitylation and proteasomal degradation. Indeed, the short intracellular half-life of LLO was extended by replacing the N-terminal lysine with stabilizing amino acids. However, the half-life extension only marginally affected cellular toxicity or virulence unless combined with mutations in the PEST-like sequence (109). Future studies should aim to identify the precise site or sites of ubiquitylation and their roles in pathogenesis and cell biology.

Consistent with the hypothesis that the LLO PEST-like sequence is important for intermolecular interactions, the PEST-like sequence contains three residues (S44, S48, and T51) that are predicted targets for MAPKs, and one or all of these residues are important for LLO phosphorylation inside of infected host cells (108). Studies on phosphorylation of the PEST-like sequence have been confounded by the observation that point mutations in the region result in increased protein production and attenuated virulence (108). For example, mutations that change the S44 codon to alanine, thereby preventing phosphorylation, have increased translation of LLO. However, mutations that change the S44 codon to other serine codons also have increased secretion and cytotoxicity—suggesting that the PEST-like sequence acts at the mRNA level to affect translation. Further evidence of translational regulation is supported by the observation that mutations in the 5' UTR alter protein expression (110,111). The unexplained effect of mutations in the PEST-like sequence on translation complicates the study of post-translational modifications in the PEST-like sequence.

In addition to the above modifications, LLO is covalently modified by exogenously- and endogenously-produced S-glutathione at its cysteine residue (112). Modification of this residue may modulate the activity of all extracellular CDCs or restrict their activity to phagosomes containing oxidoreductases. For example, this cysteine has been implicated as a target for the phagosomal thiol-reductase known as GILT (113). $GILT^{-/-}$ mice and macrophages were more resistant to *L. monocytogenes* due to a defect in phagosomal escape, presumably because LLO activity was reduced by modification with glutathione or another low molecular weight thiol. Thus, the presence of a host oxidoreductase, such as GILT, can confer cellular specificity to CDC-producing pathogens by activating CDCs in the phagosome and promoting escape. However, mutant *L. monocytogenes* in which the LLO cysteine is substituted with an alanine have a very small virulence defect.

Although a lot of work is still required to understand the role of LLO modifications inside host cells, recent work has provided a detailed mechanism describing how its N-terminus uses host cell machinery to promote LLO degradation (114). Within cells, LLO localized to puncta within the cytosol while LLO lacking the PEST-like sequence was found on the host plasma

membrane. This was due to interaction of the PEST-like sequence with the host Ap2a2 subunit of the clathrin-dependent endocytosis machinery, supporting a model in which LLO prevents cytotoxicity by accelerating the removal of LLO pores from the plasma membrane by endocytosis and targeting to autophagosomes. Interestingly, replacement of LLO PEST with the PEST-like sequence found on the human calcium receptor protein (HCaR), a G protein-coupled receptor that also interacts with Ap2a2, restored much of the virulence defect seen in a PEST deletion mutant. Though there are still some unanswered questions about the individual functions of this region of LLO, it is clear that the PEST-like sequence reduces the cytotoxicity of LLO.

1.6 Contribution of LLO to *L. monocytogenes* Pathogenesis

As discussed above, LLO has many other putative functions that are shared with CDCs produced by extracellular pathogens. Antibody to CDCs can often dramatically affect pathogenesis, as has been shown for PFO, PLY, ALO, and SLY (Bryant et al., 1993; Del Mar García-Suárez et al., 2004; Musher et al., 2001; Nakouzi et al., 2008; Takeuchi et al., 2014). In the case of *L. monocytogenes*, pretreatment of mice with 1 mg of LLO neutralizing antibody, 10-times the amount of antibody required to effectively neutralize ALO and PLY *in vivo*, resulted in reduced bacterial burden (116). However, it was later shown that this amount of antibody blocked the activity of LLO inside of cells and prevented vacuolar escape (117,118). These results suggest that LLO is required for pathogenesis of *L. monocytogenes* because it enables vacuolar escape, and that extracellular LLO has little if any effect on pathogenesis.

1.7 Future Considerations

Tissue culture models of infection provide a convenient way to study the effects that pathogens exert upon cells, and can shed insight into the host and bacterial factors required for any observed phenotypes. Bacterial mutants or antibody can be used to demonstrate the requirement for specific bacterial gene products in a given phenotype, and host mutants or specific inhibitors can be used to demonstrate host requirements. These are powerful strategies that have been used often to show the role of CDCs in the induction of host responses. For example, the conclusion that CDCs of *S. pneumoniae,* and *L. monocytogenes* induce inflammasome-dependent IL-1β secretion results from two distinct findings (1) deletion of the CDCs diminished IL-1β secretion and (2) deletion of inflammasome components also diminished IL-1β secretion from cells. However, once the host and bacterial requirements for an *in vitro* phenotype have been established, how do we accurately determine if and how the phenotype translates into an effect on pathogenesis *in vivo*?

If a host is genetically tractable and the host factor in question is nonessential, it is possible to use a similar combinatorial approach, which can appropriately be called ‘genetics-squared’ (119). For some of the proposed LLO functions, genetic models can be used to verify the role of the host factors in pathogenesis. For example, *Ubc9*$^{+/-}$ mice have been used to demonstrate the importance of SUMOylation in control of *Shigella flexneri*, which is also a facultative intracellular pathogen, and could be used similarly for *L. monocytogenes* (47). Host gene deletions were used to understand the role of IL-1β in the pathogenesis of *S. pneumoniae* and *L. monocytogenes.* Caspase-1/11$^{-/-}$ mice infected with *S. pneumoniae* had increased bacterial burdens compared to WT mice, effectively demonstrating the role of caspase-1 in control of *S. pneumoniae* (68). However, PLY-deficient strains were not used in the *in vivo* experiments and thus we are left with questions: would the PLY-deficient strain grow better than wildtype *S. pneumoniae* in WT mice as a result of not activating the inflammasome, and if so, would that

benefit still occur in caspase-1-deficient mice? These approaches are not straightforward because of the multiple effects of individual virulence factors but nevertheless should be performed whenever possible. In *L. monocytogenes*, LLO showed the same capacity for activating caspase-1 as other PFTs *in vitro*, while infection of caspase-1/11$^{-/-}$ mice yielded opposing results *in vivo* and, in our hands, had no effect on infection or immunity in mice (Sauer et al., 2011). Thus, similar tissue culture model results do not always translate directly to similar effects on pathogenesis. Furthermore, evaluating the role of LLO on caspase-1 activation—and most phenotypes for that matter—*in vivo* is difficult to assess because LLO-negative bacteria cannot grow intracellularly.

How, then, can the role of LLO *in vivo* be validated separately from its essential role in vacuolar escape? One strategy that has been used to validate the significance of extracellular CDCs to pathogenesis is the use of neutralizing antibodies. Treatment of mice with PFO- PLY-, SLY- and ALO-neutralizing antibodies prior to infection with their respective pathogens resulted in a reduction in disease, thereby providing evidence for their role in disease. Many of the proposed functions of LLO, including MAPK activation, histone dephosphorylation, mitochondrial fragmentation, Ubc9 degradation, and caspase-1 activation occur upon addition of purified LLO to cells. It has been proposed that extracellular LLO that is secreted before bacterial invasion could cause the same effects *in vivo.* We propose the following experimental process to confirm or disprove that extracellular LLO causes these effects *in vivo* and that they have an effect on pathogenesis. First, these phenotypes must be identified following infection of mice; second, administration of an LLO-neutralizing antibody must reduce or abrogate the phenotypes; and third, the administration of LLO-neutralizing antibody must affect pathogenesis.

Lastly, how can roles for cytosolic LLO be elucidated when deletion of the gene prevents phagosomal escape thereby preventing secretion of cytosolic LLO? Various approaches including inducible promoters have been used to show that LLO was necessary for cell-to-cell spread (5). An alternative approach would be to incorporate an inducible degradation tag such as the auxin-inducible degron, where LLO could be targeted for degradation in the cytosol (120,121). However, this would be very difficult to adapt to animal experiments.

The question remains, is LLO a phagosome-specific cytolysin or a multifunctional virulence factor? LLO has an abundance of features throughout its structure that allow it to mediate the escape of *L. monocytogenes* from a vacuole without causing excess cytotoxicity in the cytosol. Furthermore, it is absolutely required for disease because of its role in vacuolar escape. Thus, we believe that most evidence points to LLO being a phagosome-specific cytolysin. However, LLO may act extracellularly under some circumstances, perhaps in the intestine or during extracellular growth in the gall bladder (122). The notion that LLO can activate many of the same pathways as extracellular CDCs is intriguing, and the tools exist to validate whether or not LLO activates these pathways in the host in ways that affect the outcome of disease.

		Primary function *in vivo*	K+ dependent MAPK activation	Histone Modification	Mitochondrial fragmentation	deSUMOylation/ Ubc9 degradation	Caspase-1 activation	Macrophage TLR4 activation	Effects on cell adhesion	Protein translocation	Vacuolar Escape
CDC	**Organism**										
Listeriolysin O (LLO)	*Listeria monocytogenes*	Escape from the vacuole	(32,197)	(35)	(39)	(41)	(131,198)	(199)	(200–202)	(203)	(97)
Perfringolysin (PFO)	*Clostridium perfringens*	Disruption of neutrophil migration (71)		(35)		(41)		(199)	(74,204)		(76)
Pneumolysin (PLY)	*Streptococcus pneumoniae*	Epithelial barrier disruption (83) Transmission (205)	(206)	(35) (207)		(41,42)	(66–68,208)	(209, 210)	(211–213)		
Streptolysin O (SLO)	*Streptococcus pyogenes*	Cell killing by SPN translocation (21)	(214)			(42)	(60,215)	(199)	(213,216)	(18)	
Suilysin (SLY)	*Streptococcus suis*		(217)			(42)		(217)	(213)		
Anthrolysin O (ALO)	*Bacillus anthracis*		(218)					(199)			(219)
Vaginolysin (VLY)	*Gardnerella vaginalis*		(220)								
Other Pore-forming molecules											
T3SS				(38)	(38)		(221)			(222)	
Aerolysin	*Aeromonas hydrophila*		(32)	(37)			(58,221)				

Table 1.1 Effects of pore-forming toxins on cellular functions.

Chapter 2
An inducible Cre-*lox* system to analyze the role of LLO in *Listeria monocytogenes* pathogenesis

2.1 Summary of Results

Listeriolysin O (LLO) is the pore-forming cytolysin that allows *Listeria monocytogenes* to escape from phagocytic vacuoles and enter the host cell cytosol. LLO is expressed continuously during infection, but it has been a challenge to evaluate the importance of LLO secreted in the host cell cytosol because deletion of the gene encoding LLO (*hly*) prevents localization of *L. monocytogenes* to the cytosol. Here, we describe a *L. monocytogenes* strain (*hly*^{fl}) in which *hly* is flanked by *loxP* sites and Cre recombinase is under the transcriptional control of the *L. monocytogenes actA* promoter, which is highly induced in the host cell cytosol. In less than 2 hours after infection of bone marrow-derived macrophages (BMMs), bacteria were 100% non-hemolytic. *hly*^{fl} grew intracellularly to levels 10-fold greater than WT *L. monocytogenes* and was less cytotoxic. In an intravenous mouse model, 90% of bacteria were non-hemolytic within three hours in the spleen and eight hours in the liver. The loss of LLO led to a 2-log virulence defect in the spleen and a 4-log virulence defect in the liver compared to WT *L. monocytogenes*. Thus, the production of LLO in the cytosol has significant impact on the pathogenicity of *L. monocytogenes*.

2.2 Introduction

The field of microbial pathogenesis and the study of virulence factors has been guided for decades by Molecular Koch's Postulates, which stipulate that inactivation of a gene encoding a suspected virulence factor should lead to measurable loss of virulence, and replacement of the gene should restore pathogenicity (123). Although targeted gene deletions are invaluable in determining the function of genes and pathways, there remain circumstances in which it is not possible to generate viable deletion mutants, or deletion of a gene encoding multiple functions precludes analysis of later functions. The latter is the case for the gene encoding Listeriolysin O (*hly*) of *Listeria monocytogenes.*

LLO belongs to a large family of cholesterol-dependent cytolysins (CDCs) which also includes: Perfringolysin of *Clostridium perfringens,* Streptolysin O of *Streptococcus pyogenes,* and Pneumolysin of *Streptococcus pneumoniae* (124). Importantly, LLO is the only CDC produced by an intracellular pathogen and its roles in pathogenesis are distinct and unique compared to related CDCs (125).

Though the only clearly established role of LLO during infection is inside of *L. monocytogenes*-containing vacuoles, LLO is continuously secreted inside host cells. The continuous secretion of LLO has the potential to form pores in the host cell membrane and cause cell death. Mechanisms have been identified that suppress LLO activity within the host cytosol, lessening its potential toxicity, including: reduced activity at the neutral pH of the cytosol compared to the acidic pH of the vacuole, degradation by the proteasome, and translational repression by the 5' coding sequence (25,102,108,110). Additionally, LLO co-opts host endocytosis machinery for removal of LLO from the plasma membrane (114). Mutations that abolish any of these control mechanisms increase *L. monocytogenes* cytotoxicity (126). However, these studies do not address potential activities of LLO secreted in the cytosol.

Despite the numerous mechanisms that suppress LLO activity in the cytosol, it is still not clear whether LLO is suppressed to the point where it has no activity. Unfortunately, even the simplest questions have been difficult to address because of the early requirement for LLO, although strategies have been developed to examine this question. To show that LLO was required for escape from primary single-membrane and secondary double-membrane vacuoles, Gedde *et al.* noncovalently coupled purified LLO to Δ*hly L. monocytogenes* and observed that

the bacteria were able to escape from the primary vacuole, but because of their inability to produce more LLO they became trapped in secondary vacuoles (4). Similarly, Dancz *et al.* demonstrated that *L. monocytogenes* expressing IPTG-inducible LLO remain trapped in vacuoles until addition of IPTG (5). Czuczman *et al.* studied *L. monocytogenes* in HeLa cells, in which LLO is not required for escape from the vacuole, and concluded that LLO causes localized plasma membrane damage that allows *L. monocytogenes* to hijack the cell efferocytosis machinery for cell-to-cell spread (127). Others have observed that LLO disrupts SUMOylation, modifies histones, and causes mitochondrial fragmentation during infection by simply adding purified LLO exogenously to cells (29,30,35,39,42). These studies have all tried to circumvent the requirement for LLO in the vacuole, but the diversity in the techniques used and their limitations makes it a challenge to integrate the conclusions into a complete picture. Additionally, these studies have not addressed the effects of these activities in mice.

We have developed a tool for studying LLO that does not circumvent the requirement for LLO in vacuolar escape. We engineered a strain of *L. monocytogenes* that initially produces LLO, allowing it to escape from the vacuole. After escape from the vacuole *hly* is excised by Cre-*lox*-mediated DNA recombination and the strain becomes a Δ*hly* mutant. Here we report rapid excision of *hly* in bone marrow-derived macrophages (BMMs) and in a mouse model. In BMMs, LLO secreted in the cytosol contributes to significant amounts of cytotoxicity. *hly*^{fl} grew to a level 10-fold higher than WT *L. monocytogenes* in BMMs, likely due to reduced cell death.

2.3 Results

2.3.1 Cre-*lox* allows for rapid excision of *hly* during infection of macrophages

To study the role of LLO secreted in the cytosol during infection, we engineered a strain of *L. monocytogenes*, called *hly*^{fl}, to excise the gene encoding LLO, *hly*, following escape of *L. monocytogenes* from the phagocytic vacuole. Specifically, *loxP* sites were inserted into the *L. monocytogenes* chromosome to flank *hly* and an adjacent gene, *tetL*, which provides tetracycline resistance. Cre recombinase, which mediates DNA recombination between *loxP* sites, was inserted into the chromosome using the pPL2 integrative vector, and expressed under the control of the *L. monocytogenes actA* promoter, which is relatively inactive prior to vacuolar escape of *L. monocytogenes* and becomes highly expressed in the cytosol (Figure 1A). Thus, this strain is able to produce LLO initially to facilitate escape from the phagocytic vacuole but, once in the cytosol, *hly* is excised and LLO production ceases. To determine the efficiency of the system, BMMs were infected with *hly*^{fl} *L. monocytogenes* and bacteria from the infected cells were recovered at different time points and plated on blood-agar media. Secreted LLO causes rapid β-hemolysis and *L. monocytogenes* colonies that secrete LLO can be easily identified (Figure 1B). Prior to infection, *hly*^{fl} L. monocytogenes were grown in broth containing tetracycline to select against low-level excision of *hly* and *tetL*. By 30 minutes post-infection almost 90% of recovered CFU were non-hemolytic (Figure 1C). By 60 minutes post-infection, 98% of recovered CFU were non-hemolytic and by 90 minutes post-infection all colonies were non-hemolytic. Therefore, the excision of *hly* is rapid and complete during infection of BMMs.

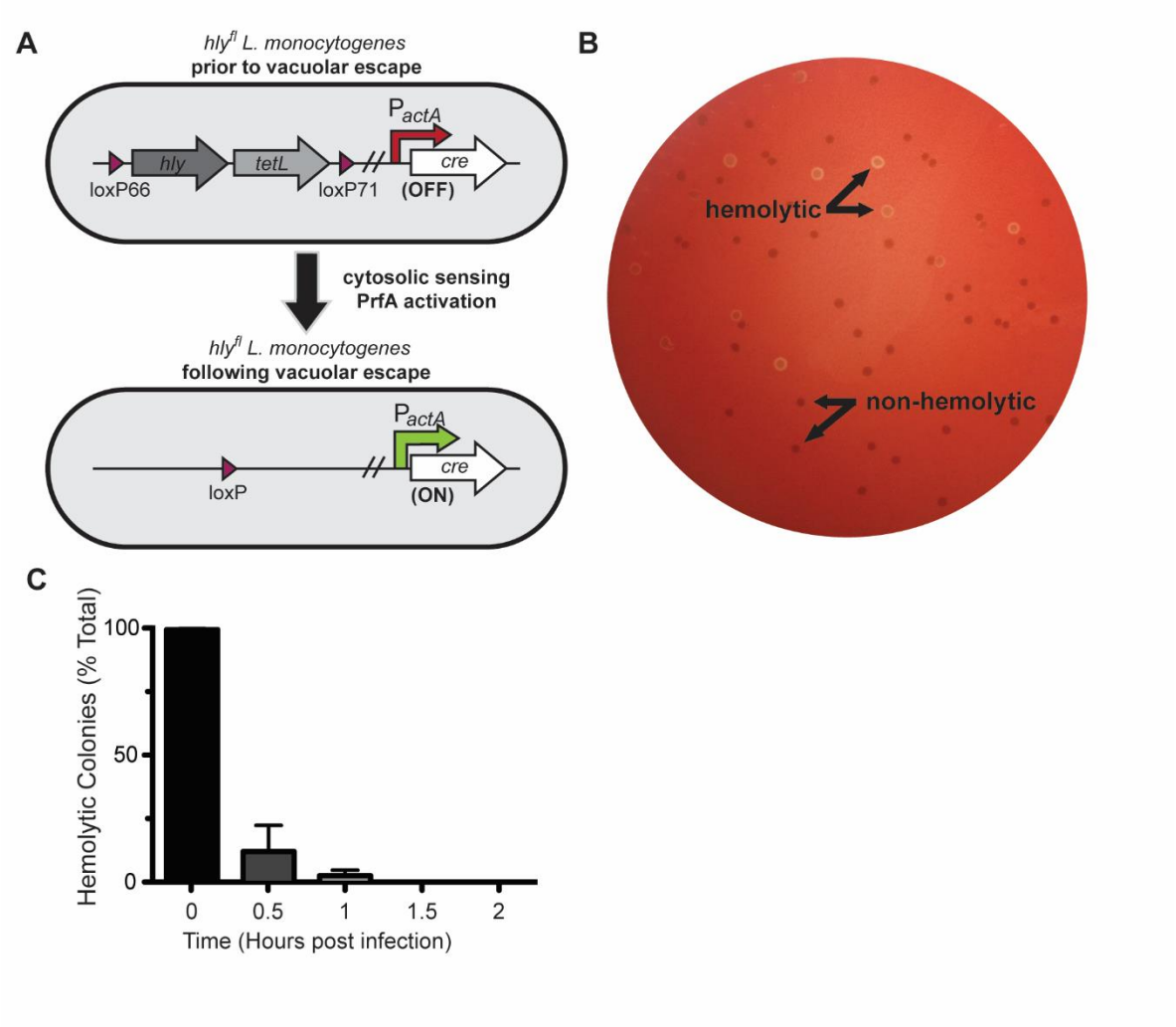


Figure 2.1. *hly* is excised in the *hly*^{fl} strain in BMMs through DNA recombination.

(A) Schematic of *hly*^{fl} *L. monocytogenes* strain. The *hly* and *tetL* (tetracycline resistance) genes are flanked by *loxP* sites. Cre recombinase is controlled by the *actA* promoter. Recombination between *loxP* sites leads to the excision of the DNA encoding *hly* and *tetL*; (B) Hemolytic and non-hemolytic colonies of *hly*^{fl} (C) To quantify the excision of hly, BMMs were infected with *hly*^{fl} and both hemolytic and non-hemolytic colonies were enumerated by plating bacteria on blood-agar media at different timepoints. Mean and SD of data pooled from three independent experiments are shown. (A and C) Reprinted from Cell Host & Microbe, Vol 23/Issue 6, Chen Chen, Brittney N. Nguyen, Gabriel Mitchell, Shally R. Margolis, Darren Ma, Daniel A. Portnoy, The Listeriolysin O PEST-like Sequence Co-opts AP-2-Mediated Endocytosis to Prevent Plasma Membrane Damage during Listeria Infection, 786-795., Copyright (2018), with permission from Elsevier.

2.3.2 LLO secreted in the cytosol affects intracellular growth and contributes to cytotoxicity

To determine whether secretion of LLO by *L. monocytogenes* in the cytosol affects the growth of the bacteria in cells, intracellular growth was evaluated in BMMs. During the first five hours of infection, *hly*^{fl} *L. monocytogenes* grew identically to WT *L. monocytogenes* and Δ*actA*, which is defective in actin-based motility and therefore defective in cell-to-cell spread (Fig 2A). However, after five hours of infection the growth of the strains diverged. Between five and twenty-four hours of infection, the number of WT *L. monocytogenes* plateaued and then declined, but the number of recovered *hly*^{fl} *L. monocytogenes* increased to 10-fold more than the maximum of WT and remained elevated, suggesting that secretion of LLO in the cytosol negatively impacts growth of WT.

WT *L. monocytogenes* has the ability to spread to and replicate in neighboring cells. If *hly*^{fl} has a defect in escape from secondary vacuoles that would limit its ability to replicate in neighboring cells, the difference in growth between WT and *hly*^{fl} may reflect both the effects of LLO secreted in the cytosol and growth following cell-to-cell spread. To analyze the effects of LLO in the cytosol without complication by cell-to-cell spread, we compared growth of *hly*^{fl} in a Δ*actA* background (Δ*actA hly*^{fl}) to Δ*actA*, which is defective in actin-based motility and therefore defective in cell-to-cell spread. During the first eight hours of infection, Δ*actA* and Δ*actA hly*^{fl} grew similarly to WT. However, between eight and twenty-four hours, the number of Δ*actA* bacteria decreased dramatically, whereas Δ*actA hly*^{fl} decreased much less – having as much as 100-fold more bacteria than Δ*actA*. The rapid loss of Δ*actA* CFU, which is due to the influx of gentamicin (128), was partially rescued by deletion of *hly* in the cytosol, indicating that the decline of Δ*actA* in cells is largely LLO-dependent.

The ability of LLO to form pores in cholesterol-containing cell membranes is well documented. We hypothesized that the growth of WT *L. monocytogenes* could be restricted by LLO-induced cytotoxicity because LLO has the potential to bind to the cell membrane and cause cell death. To quantify the amount of cell death caused by *L. monocytogenes* infection, cytotoxicity was measured by LDH release assay (Fig 2B). After a 24-hour infection of BMMs, 43% of cells were killed by WT *L. monocytogenes* infection. Only 22% of cells were killed by *hly*^{fl} *L. monocytogenes* infection, indicating that LLO secreted in the cytosol contributes significantly to cytotoxicity during infection.

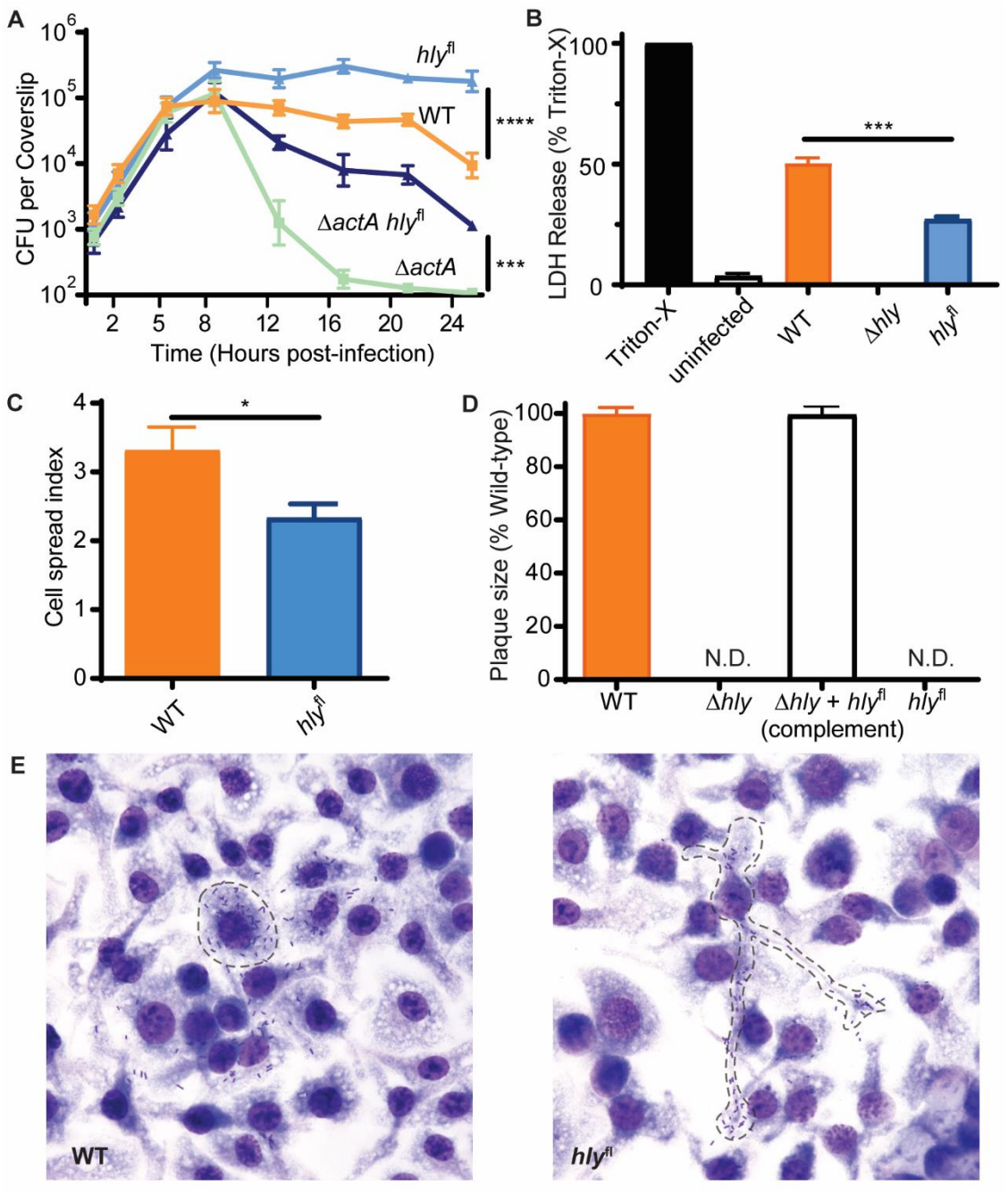


Figure 2.2. *hly*^{fl} grew intracellularly and had reduced cytotoxicity but had a defect in cell-to-cell spread.

(A) BMMs were infected at an MOI of 0.17 with *L. monocytogenes* and intracellular CFU were enumerated at different times. Three biological replicates were measured in each experiment, and experimental results were combined. For Δ*actA* and Δ*actA hly*^{fl} n=6. For WT and *hly*^{fl} n=9. Mean and SEM are shown. Results were log-transformed and means were

compared using Holm Sidak's multiple comparisons test. (B) BMMs were infected with *L. monocytogenes* at a MOI of 4 for 24 hours. LDH release was measured and values were normalized according to lysis with 1% Triton-X 100 representing 100% LDH release and the condition with the smallest mean value representing 0% LDH release. The average of three technical replicates per experiment, from three independent experiments are presented. Means were compared using an unpaired t-test. (C) BMMs were infected with *L. monocytogenes* and cell-to-cell spread was quantified 5 hours post infection. WT n=67, *hly*^{fl} n=60. Means were compared using an unpaired t-test. (D) L2 cells were infected with *L. monocytogenes* at a MOI of 0.1. Plaque size was measured after 3 days and values were normalized to WT plaque size. Mean and SEM for data pooled from three independent experiments is shown. WT n=19, Δ*hly* + *hly*^{fl} n=14. N.D. indicates no plaques were detectable. (E) Representative images of the BMMs infected with *L. monocytogenes* for cell-to-cell spread analysis in (C). Dashed lines are used to indicate the initially-infected cells.

2.3.3 Cell death caused by LLO is not apoptosis or necroptosis

When the impacts of cell-to-cell spread on growth in BMMs were removed by comparing Δ*actA* to Δ*actA hly*^{fl}, deletion of *hly* in the cytosol still provided a growth advantage. The results indicate that WT *L. monocytogenes* has a growth defect due to LLO-dependent cytotoxicity. To determine the type of cytotoxicity caused by LLO, BMMs were infected with WT and *hly*^{fl} *L. monocytogenes* and treated with the pan-caspase inhibitor zVAD-fmk, which blocks apoptotic cell death, or Necrostatin-1, which blocks necroptosis (Fig 2.3A). Treatment of infected BMMs with zVAD-fmk or Necrostatin-1 did not reduce LDH release caused by WT *L. monocytogenes* infection. Surprisingly, zVAD-fmk treatment of macrophages infected with WT or *hly*^{fl} *L. monocytogenes* resulted in significantly increased cell death and reduced growth in BMMs (Fig2.3A-B). To determine the type of cell death caused by infection in combination with zVAD-fmk treatment, zVAD-fmk was used in combination with Necrostatin-1. Necrostatin-1 treatment significantly reduced zVAD-fmk-dependent cytotoxicity (Fig 2.3A), suggesting that zVAD-fmk treatment leads to necroptosis of *L. monocytogenes*-infected BMMs. To confirm that zVAD-fmk leads to necroptosis, Caspase-1/11$^{-/-}$ and Rip3k$^{-/-}$ BMMs were infected in the presence and absence of zVAD-fmk. Cytotoxicity was significantly increased by zVAD-fmk in Caspase-1/11$^{-/-}$ BMMs (Fig 2.3C), indicating that zVAD-fmk did not lead to pyroptotic cell death. Additionally, WT *L. monocytogenes* infection resulted in significantly higher LDH release than *hly*^{fl} *L. monocytogenes* in Caspase-1/11$^{-/-}$ BMMs, similar to WT BMMs. However, zVAD-fmk treatment did not result in increased cell death in Ripk3$^{-/-}$ BMMs (Fig 2.3D). Together, our results suggest that LLO-mediated cytotoxicity is not apoptosis, necroptosis, or pyroptosis, and that zVAD-fmk treatment in combination with *L. monocytogenes* infection leads to necroptosis.

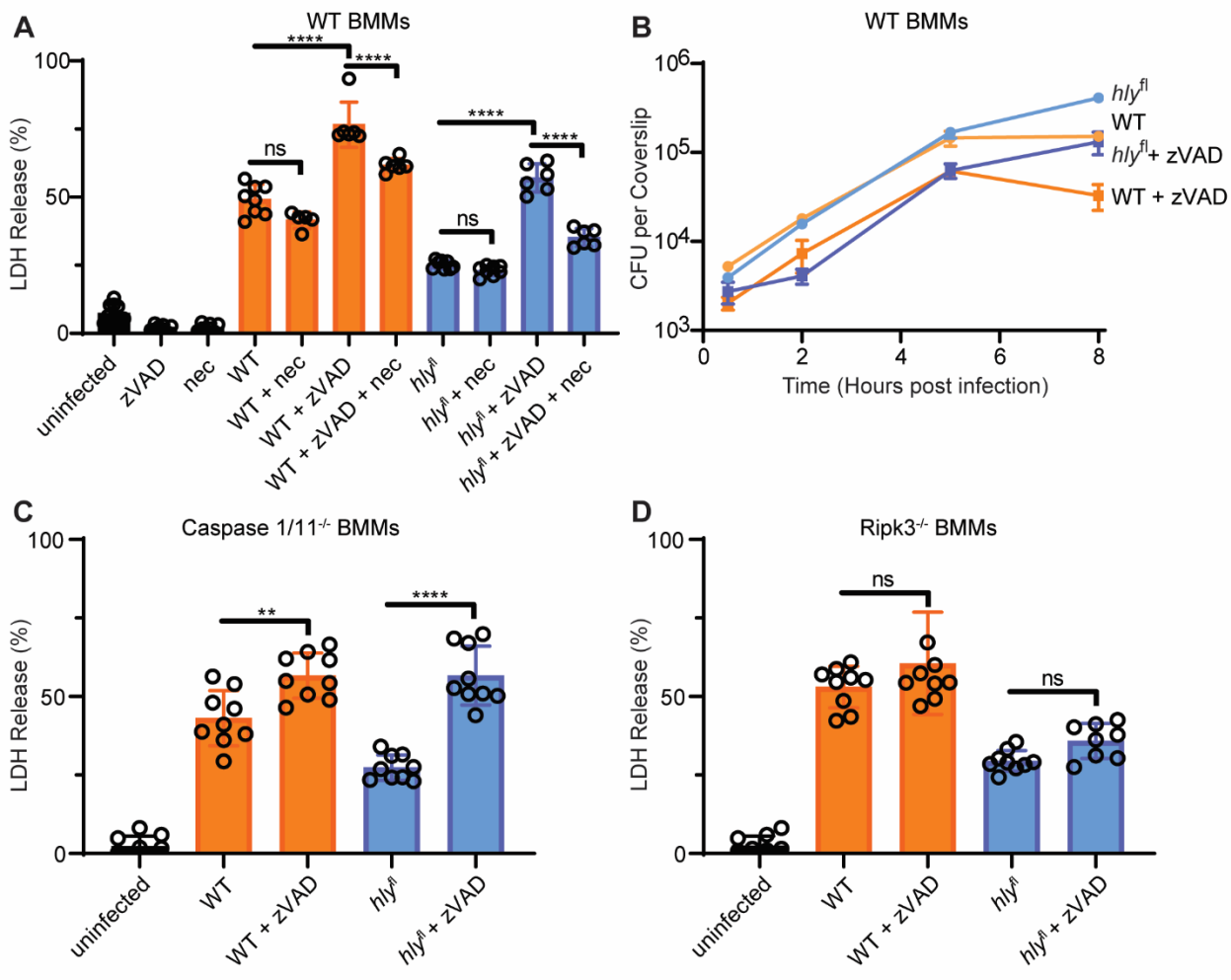


Figure 2.3. Inhibition of caspases results in increased cell death during infection.

WT BMMs (A), Caspase-1/11$^{-/-}$ BMMs (C) or Ripk3$^{-/-}$ BMMs (D) were infected with *L. monocytogenes* at a MOI of 4 for 24 hours. (B) BMMs were infected at an MOI of 0.17 with *L. monocytogenes* and intracellular CFU were enumerated at different times. Data are pooled from two independent experiments. For cell death inhibitors, cells were pretreated for 1 hour with 10 μM zVAD-fmk (zVAD) and/or 50 μM Necrostatin-1 (nec). LDH release was measured and values were normalized according to lysis with 1% Triton-X 100 representing 100% LDH release and the uninfected representing 0% LDH release. Mean and SEM for data pooled from 2-3 independent experiments is shown. Data were analyzed using a Holm-Sidak Multiple Comparisons test.

2.3.4 *hly* is excised *in vivo* and its excision reduces virulence

To quantify the efficiency of the *hly*^{fl} Cre-*lox* system *in vivo*, C57BL/6J mice were infected intravenously with 10^5 CFU of *hly*^{fl} *L. monocytogenes* (Fig 2.4). Hemolytic capacity of the inoculum was verified by plating on blood agar. At 1, 2, 3, 5, 8, and 24 hours post-infection, bacteria were recovered from the spleen and liver and plated on blood agar and both hemolytic and non-hemolytic CFU were enumerated. In the spleen (Fig 2.4A), less than 20% of bacteria were hemolytic one hour post-infection. Hemolytic bacteria represented less than 2% of the population three hours post-infection and were nearly undetectable five hours post-infection. 24 hours post infection, a small population of hemolytic bacteria were detected in the spleen (see discussion). In the liver (Fig 2.4B), excision of *hly* was slower than in the spleen. One hour post-infection, only 35% of bacteria were non-hemolytic; eight hours post-infection 95% of bacteria were non-hemolytic and hemolytic colonies were undetectable by 24 hours post-infection.

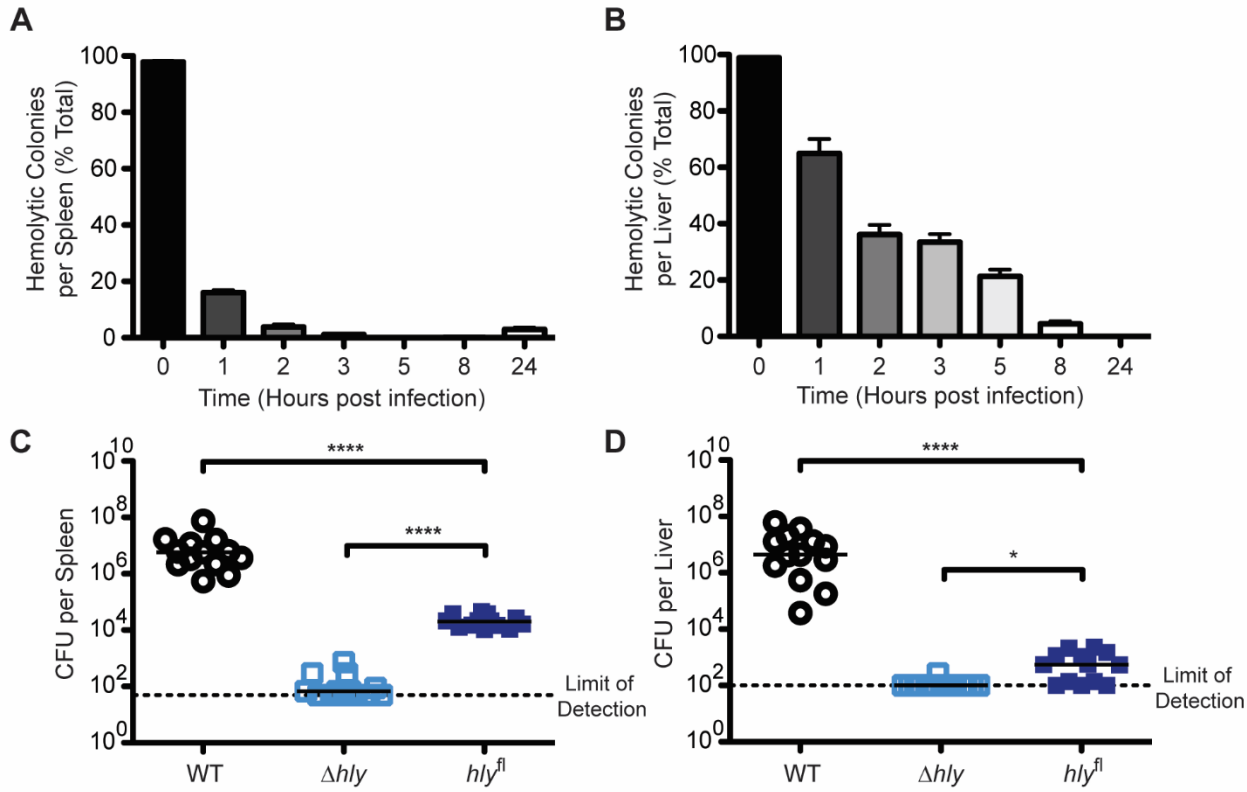


Figure 2.4. *hly*^{fl} recombines *in vivo* and is attenuated in mice.

(A and B) C57BL/6J mice were infected with 1 x 10^5 CFU of *hly*^{fl} *L. monocytogenes.* For each timepoint, hemolytic and non-hemolytic colonies recovered from the (A) spleen and (B) liver were enumerated. % Total Hemolytic colonies were calculated for each mouse. Mean and SEM are shown. Data are pooled from two independent experiments. For each timepoint, n=6. (C and D) CD-1 mice were infected with 1 x 10^5 CFU of *L. monocytogenes.* 48 hours post-infection, CFU from the (C) spleen and (D) liver were enumerated. Data are pooled from three independent experiments and medians are shown. n=13. Means were compared using Holm Sidak's multiple comparisons test.

To determine the importance of LLO after escape of *L. monocytogenes* from the initial vacuole, we characterized *hly*^{fl} *L. monocytogenes* using a mouse model of virulence. CD-1 mice were infected intravenously with 10^5 CFU of *L. monocytogenes* (Fig 2.4). WT *L. monocytogenes* grew to 10^7 CFU in both the spleen and liver, while Δ*hly L. monocytogenes* was extremely attenuated with bacteria from the spleen and liver near or below the limit of detection in most mice. Interestingly, *hly*^{fl} *L. monocytogenes* had a moderate level of attenuation. 10^4 CFU were recovered from the spleen, representing a statistically-significant 3-log reduction in virulence compared to WT, although a smaller reduction in virulence than that of Δ*hly* (Fig 2.4C). In the liver, less than 10^3 CFU were recovered, similar to Δ*hly* (Fig 2.4D).

2.3.5 Vaccination with *hly*^{fl} confers protective immunity

The two requirements for a vaccine are safety and efficacy. In Figure 3, we showed that *hly*^{fl} is highly attenuated, and thus satisfies the safety requirement. To test the efficacy of *hly*^{fl} *L. monocytogenes* as a vaccine, protection of *hly*^{fl} was compared to protection conferred by Δ*actA*, which is well-established as an attenuated and effective vaccine strain (129), and Δ*hly*, which does not confer strong protection. C57BL/6J mice were vaccinated with either 10^3 or 10^5 *hly*^{fl}, 10^3 or 10^5 Δ*actA*, or 10^8 Δ*hly*. Four weeks post-vaccination, the mice were challenged with a lethal dose of WT *L. monocytogenes*. Three days post-challenge, CFU from the spleen were enumerated (Fig 2.5A). Vaccination with 10^5 CFU of *hly*^{fl} provided 5-logs of protection, albeit less protection than Δ*actA*, while vaccination with 10^5 CFU of Δ*hly* did not confer protection. However, vaccination with 10^3 *hly*^{fl} was not as protective as 10^3 Δ*actA*.

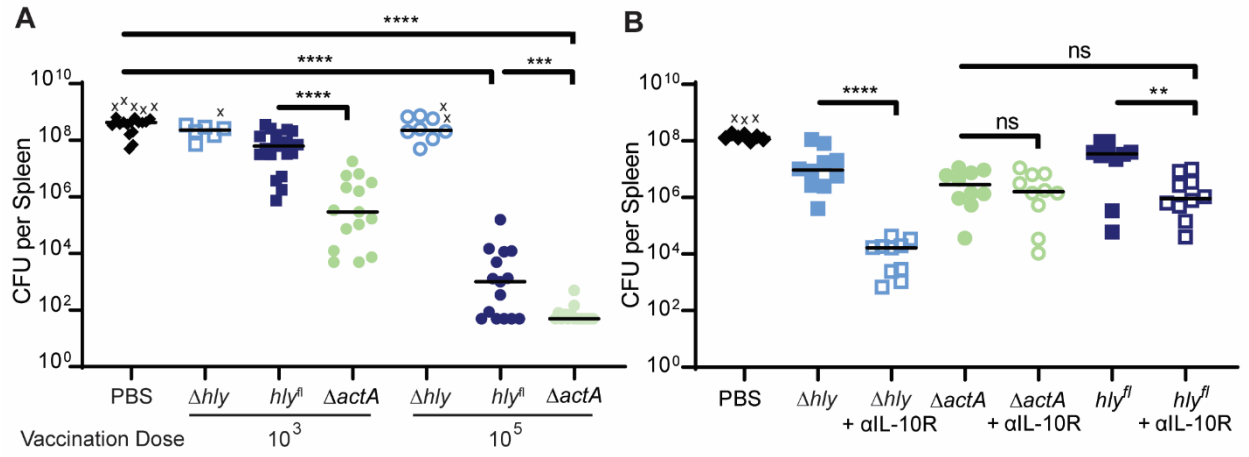


Figure 2.5. Vaccination with *hly*^{fl} confers protective immunity in mice.

(A) C57BL/6J mice were vaccinated with 10^3 or 10^5 CFU of *L. monocytogenes.* 4 weeks post-vaccination, mice were challenged with 5 x 10^4 – 1 x 10^5 CFU of WT *L. monocytogenes.* Three days post-challenge, CFU were enumerated. Data are pooled from 2-3 independent experiments. (B) C57BL/6J mice were vaccinated with 10^3 Δ*actA* or *hly*^{fl} *L. monocytogenes* or 10^8 Δ*hly L. monocytogenes* +/- treatment with αIL-10R antibody. Mice were challenged with 5 x 10^4 CFU of WT *L. monocytogenes.* Three days post-challenge, CFU were enumerated. Data are pooled from 2 independent experiments. Data were analyzed using a Holm-Sidak Multiple Comparisons test. X indicates a mouse that died from the WT challenge.

As vaccination with vacuole-confined Δ*hly* is inhibited by the secretion of the immunosuppressive cytokine IL-10 (130), we hypothesized that *hly*^{fl} is also inhibited by IL-10 because it is defective in cell-to-cell spread and remains confined in secondary vacuoles. To test this hypothesis, we performed protection studies using an IL-10 receptor blocking antibody (αIL-10R), which improves the protective capacity of Δ*hly* (130). Indeed, administration of αIL-10R improved the protective capacity of Δ*hly* and *hly*^{fl} but not Δ*actA* L. monocytogenes (Fig 2.5B). Furthermore, the protection conferred by *hly*^{fl} was improved to levels similar to those conferred by vaccination with Δ*actA*. Thus, *hly*^{fl} is a highly attenuated strain of L. monocytogenes capable of inducing protective immunity, though its protection is reduced compared to Δ*actA* likely due to IL-10.

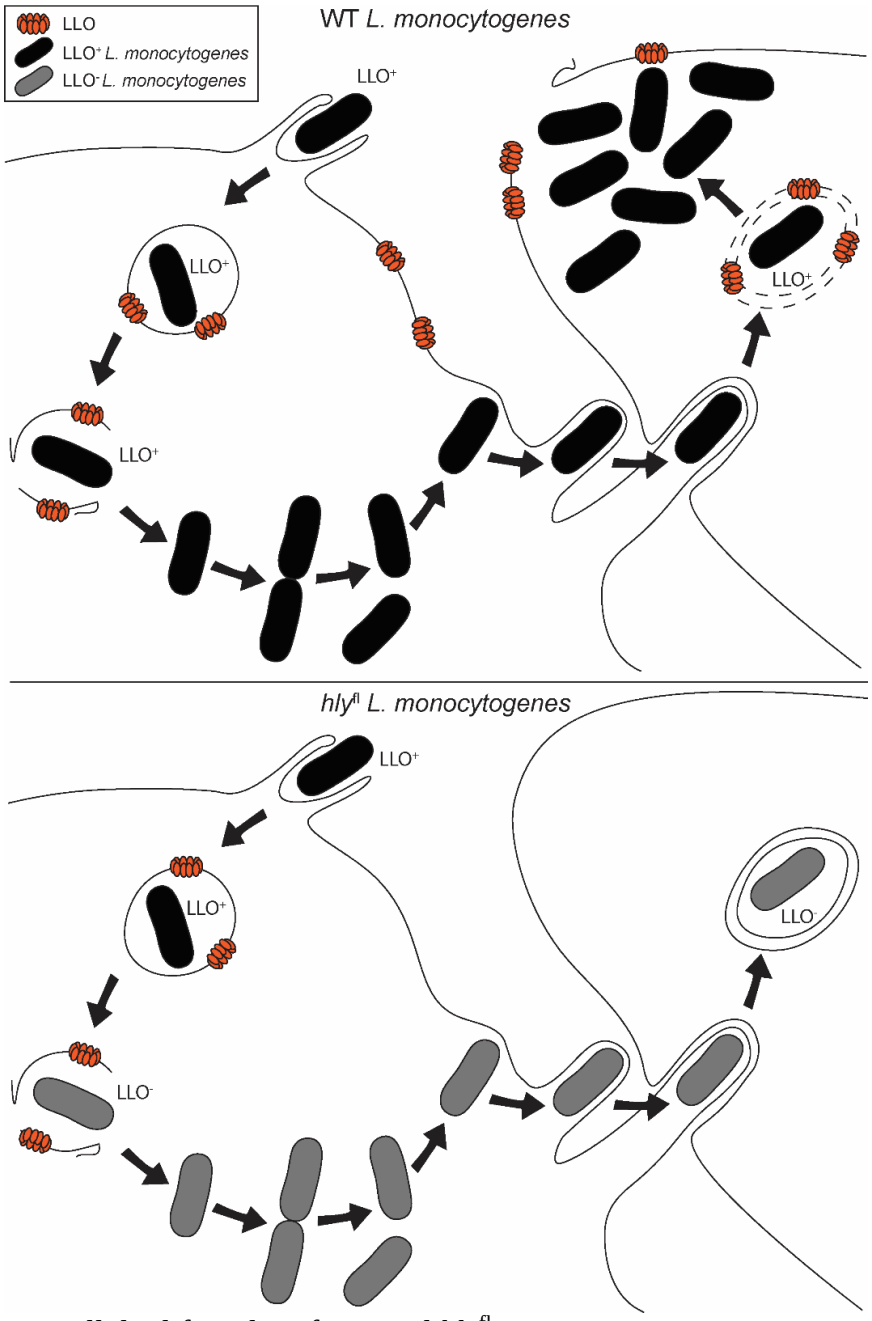


Figure 2.6. Intracellular lifecycles of WT and *hly*^{fl} *L. monocytogenes.*

WT *L. monocytogenes* enters cells and secretes LLO, allowing it to escape phagocytic vacuoles and enter the cytosol. In the cytosol, the bacteria replicate to high numbers and continuously secrete LLO. *L. monocytogenes* uses actin-based motility to form membrane protrusions, which are engulfed by neighboring cells and resolved into double-membraned vacuoles. LLO facilitates bacterial escape from secondary vacuoles and the cycle continues. *hly*^{fl} *L. monocytogenes* enters cells and secretes LLO, allowing bacterial entry into the cytosol. In the cytosol, Cre-recombinase expression is upregulated and *hly* is deleted. Bacteria in the cytosol replicate to high numbers but do not produce LLO. *hly*^{fl} *L. monocytogenes* spreads to neighboring cells but remains trapped in secondary vacuoles.

2.4 Discussion

As a member of the cholesterol-dependent cytolysin (CDC) family of pore-forming toxins, LLO is unique as the only CDC that is secreted by an intracellular pathogen and therefore the only CDC that primarily acts on cells from within. Therefore, LLO likely affects cells in ways that extracellular CDCs do not. Yet, because deletion of *hly* prevents study of the effects of LLO in the cytosol, other studies have utilized the application of exogenous purified LLO to study its effects (29,30,35,39,42). Here we described a L. monocytogenes strain that uses a Cre-*lox* system to delete *hly* following vacuolar escape. This strain, hly^{fl}, became 100% non-hemolytic less than 1.5 hours after infection of macrophages (Fig 1C) and replicated to high numbers in the cytosol (Fig 2A and 2E). *hly* excision after vacuolar escape allows the study of LLO functions in the cytosol separate from its role in vacuolar escape.

2.4.1 Insights into the effects of LLO during infection

LLO is a pore-forming toxin that oligomerizes and forms pores in cholesterol-containing membranes, including the host cell vacuolar and plasma membranes (9,10). Secretion of LLO in the cytosol has the potential to damage the host cell plasma membrane. Although multiple mechanisms limit LLO damage to the cell plasma membrane, it has been difficult to establish whether these mechanisms are entirely effective in preventing cell death (125). Here we showed that *L. monocytogenes* that does not produce LLO in the cytosol replicates to greater numbers in macrophages and is less cytotoxic. The revelation that LLO secreted in the cytosol is cytotoxic makes it more curious that *L. monocytogenes* continuously secretes LLO. It is possible that the continuous production of LLO is to ensure a rapid escape from secondary vacuoles, as defects that reduce cell-to-cell spread are highly attenuated.

It is also possible that the innate immune response to LLO-induced cell death contributes to pathogenesis. In the host cell cytosol, infrequent lysis of *L. monocytogenes* induces pyroptotic cell death via the AIM2 inflammasome (131). Some forms of cell death are thought to inhibit the generation of protective immunity. Strains of *L. monocytogenes* engineered to induce pyroptosis, necrosis or apoptosis inhibit the generation of protective immunity (132). Thus, it is likely that LLO-induced cell death also affects the immune response to *L. monocytogenes* infection. Our results indicated that LLO does not lead to apoptosis, necroptosis, or pyroptosis. Therefore, cell death caused by LLO may be a different type of programmed cell death or, more likely, unprogrammed necrosis.

Strikingly, we observed that inhibition of caspases with the pan-caspase inhibitor zVAD-fmk resulted in necroptotic cell death during *L. monocytogenes* infection. The apoptosis and necroptosis cell death pathways are tightly connected, such that induction of apoptosis prevents necroptosis (133). Many of the signals that induce apoptosis can also induce necroptosis, such as activation of TNF receptors. As a result, caspase inhibition with zVAD-fmk in cells stimulated with TNF, for example, can lead to necroptosis (133). In mice, *L. monocytogenes* causes rapid necroptosis of Kupffer cells in the liver, which promotes clearance of the infection (134). It has been suggested that necroptosis is a host immune strategy that is protective against *L. monocytogenes* infection (135). Our results suggest that, in BMMs, *L. monocytogenes* infection activates caspases that prevent necroptotic cell death. However, *L. monocytogenes*-infected BMMs do not die by apoptosis (136). Therefore, it is possible that *L. monocytogenes* has a mechanism for activating apoptotic caspases to prevent necroptosis, while also inhibiting the complete execution of the apoptotic cell death pathway, as an intracellular survival strategy. How *L. monocytogenes* promotes cell survival merits further investigation.

Analysis of the kinetics of *hly* deletion, specifically the more rapid deletion in the spleen compared to the liver, revealed differences in the environments experienced by *L. monocytogenes*. CD169+ macrophages that are localized to the marginal zone of the spleen and dendritic cells are thought to be the first splenic cell types infected by *L. monocytogenes* upon intravenous inoculation (137,138). Three hours post infection, the majority of *L. monocytogenes* in the spleen are trapped within CD169+ macrophages (137). Following infection with *hly*^{fl}, bacteria in the spleen became non-hemolytic at a rate similar to in BMMs, with almost complete loss of hemolytic capacity three hours post-infection, suggesting that infection of BMMs closely models infection in the spleen with respect to activation of virulence genes. However, bacteria became non-hemolytic at a much slower rate in the liver, with greater than 20% of bacteria remaining hemolytic at 5 hours post-infection and complete loss of hemolysis between eight and 24 hours post-infection. In the liver, four hours after infection with *L. monocytogenes* 100% of infected cells are tissue-resident macrophages, known as Kupffer cells (134). Bacteria that are not killed by the Kupffer cells can transfer to hepatocytes, which become heavily infected (139). Over the next couple days, infected Kupffer cells die by necroptosis; infiltrating neutrophils and monocyte-derived macrophages lyse infected hepatocytes and become the primarily infected cells (139–141). It is possible that *hly*^{fl} became non-hemolytic slowly in the liver because *actA* expression was not efficiently upregulated in Kupffer cells, and Cre-*lox* recombination only occurred after the bacteria were transferred to hepatocytes, neutrophils and/or monocyte-derived macrophages. If this is the case, it would be interesting to understand why the intracellular environment of a Kupffer cell does not activate *actA* expression like other cells. Alternatively, the bacteria that became non-hemolytic during the first 8 hours of infection could represent the population of bacteria inside Kupffer cells, hepatocytes and/or infiltrating neutrophils and monocyte-derived macrophages, and the bacteria that remained hemolytic may represent an extracellular population of bacteria in the liver. A small population of extracellular bacteria associated with nonparenchymal cells in the liver six hours after infection has been previously identified, though it is not clear whether these bacteria became extracellular following lysis of infected hepatocytes, or whether they never infected cells (142).

2.4.2 Limitations of *hly*^{fl}

Although we have successfully employed *hly*^{fl} to demonstrate the contribution of LLO to cytotoxicity in macrophages, this system is limited by the fact that loss of LLO is permanent and not conditional to the environment of the cytosol. As a result, *hly*^{fl} became trapped in secondary vacuoles and was defective in cell-to-cell spread (Fig 2C-E). Bacteria that are released from the cytosol upon cell lysis also behave like LLO-minus mutants. The inability to escape subsequent vacuoles likely explains the attenuation of *hly*^{fl} in mice (Fig 3C and 3D), and why *hly*^{fl} vaccination was improved by αIL-10R antibody (Fig 4B).

An additional complication of *hly*^{fl} is that Cre-*lox* recombination is susceptible to inactivation. A previous study in which a transposon library was generated in a strain of *L. monocytogenes* with Cre-*lox* identified transposon insertions in the *actA* promoter driving *cre* expression and *loxP* sites that prevented recombination. We observed a population of hemolytic bacteria in the spleen that expanded between eight- and 24 hours post-infection. We isolated several colonies of hemolytic bacteria 24 hours post-infection and reinfected BMMs, and bacteria were 100% hemolytic five hours post-infection (data not shown). It is possible that these hemolytic bacteria are the progeny of a founding bacterium that had a mutation in its Cre-*lox* machinery that prevented recombination from occurring.

In the future, the ideal tool to study the cytosolic effects of LLO in mice would not secrete LLO in the host cell cytosol but could secrete LLO upon entry into secondary cells to continue the life cycle. Nevertheless, we believe *hly*^{fl} is well-suited for studying LLO secreted in the cytosol in cells, and insights gained from study of *hly*^{fl} in cells can be translated to the whole animal setting using various mouse models.

2.5 Conclusion

Cre-*lox* recombination has been a popular tool for the study of plants and mice for many decades (143–145). In bacteria its use has been more limited, and it has only been used a few times in *L. monocytogene*s. Previously in *L. monocytogenes*, Cre-*lox* was used to generate a strain that cannot replicate following activation of the *actA* promoter by flanking essential genes near the origin of replication with *loxP* sites and driving Cre expression with the *actA* promoter (146). This strain is highly attenuated and potently activates the CD8+ T-cell response and thus is a candidate vaccine-delivery system (147). In another instance, a strain of L. monocytogenes that deletes *actA* in the host cell cytosol was used to show that ActA expressed in the host cell cytosol contributes to cell-to-cell spread and simultaneously allows L. monocytogenes to avoid xenophagy (148). This works represents the first use of Cre-*lox* recombination to study the function of a virulence factor that is active at temporally and spatially distinct periods. We believe that this system has the promise to uncover many effects of LLO secreted in the cytosol and could also uniquely contribute to better understanding the cellular responses to membrane damage from an intracellularly secreted pore-forming toxin.

2.6 Materials and Methods

Construction of *hly*^{fl}

hly^{fl} was constructed by integrating two plasmids, one encoding *hly* and *tetL* flanked by *loxP* sites and the other encoding cre downstream of the *actA* promoter, into a Δ*hly* strain of *L. monocytogenes.* The *hly* and *tetL* genes were cloned into pPL1 and flanked by *lox66/lox71 loxP* sites such that Cre expression resulted in excision of the region flanked by *loxP* sites (fl). The resulting plasmid (pPL1-*hly*^{fl}) was transformed into SM10 E. Coli. The cre recombinase gene was previously engineered downstream of the *actA* promoter in pPL2e, yielding the plasmid pPL2e-*actA*-*cre*, which was also transformed into SM10 E. Coli. Transconjugation was performed to integrate both plasmids into Δ*hly L. monocytogenes* in a stepwise manner. First, pPL1-*hly*^{fl} was transconjugated with Δ*hly L. monocytogenes* and transconjugate colonies that were resistant to streptomycin (200μg/mL) and chloramphenicol (7.5 μg/mL) were selected. Second, Δ*hly* pPL1-hlyfl was transconjugated with pPL2e-*actA*-*cre*, and transconjugate colonies resistant to streptomycin, chloramphenicol, and erythroymycin (1 μg/mL) were selected. Similarly, Δ*actA hly*^{fl} was engineered by transconjugating pPL1-*hly*^{fl} and pPL2e-actA-cre into Δ*actA*Δ*hly*. The *hly*^{fl} complement strain was engineered by transconjugating Δ*hly* with pPL1-*hly*^{fl}.

A control pPL1 plasmid (pPL1-*tetL*^{fl}) encoding *tetL*, but not *hly*, flanked by *loxP* sites was engineered by excising *hly* from pPL1-*hly*^{fl}. The Δ*hly* control strain was engineered by transconjugating Δ*hly* with pPL1-*tetL*. The WT control strain was engineered by transconjugating WT with pPL1-*tetL*^{fl}. The Δ*actA* control strain was engineered by transconjugating Δ*actA* with pPL1-*tetL*^{fl}.

Bacterial Culture

Bacteria were grown overnight at 37°C in BHI containing 200μg/mL streptomycin, and bacteria with *tetL* were additionally grown in 2 μg/mL tetracycline. Overnight cultures were diluted 1:200 and grown in BHI containing streptomycin (for bacteria without *tetL*) or streptomycin and tetracycline (for bacteria with *tetL*) at 37°C, shaking, to an optical density of 0.5. These cultures were then pelleted by centrifugation, and resuspended in phosphate-buffered solution (PBS) containing 9% glycerol. These cultures were then aliquoted and frozen at -80°C. Aliquots were thawed and used directly for experiments.

Preparation of M-CSF

3T3 cell media was prepared using DMEM with 10% FBS, 1% L-Glutamine, and 1% Sodium pyruvate, with or without 1x Penicillin Streptomycin Solution ("Pen/Strep"; Corning). 10^7 M-CSF-producing 3T3 cells were seeded into a T75 flask with 20mL media containing Pen/Strep and grown at 37°C 5% CO_2 (Day 1). To split cells, media was aspirated, cells were washed with warm PBS, and incubated with 0.05% Trypsin-EDTA (Gibco) for five minutes at 37°C. On day 4, cells were split to a T225 in 50mL media containing Pen/Strep. On day 7, cells were split to five T225 flasks in media without Pen/Strep. On day 9 or 10, when cells when cells were 100% confluent, the five T225 flasks were split into 25 T225 flasks and grown until 100% confluent (about 3 days) and an additional two days (about 5 days total) in media without Pen/Strep. On day 14 or 15, supernatant was removed from all flasks, filter sterilized with a 0.2μM bottle filter, and stored at 4°C. 50mL fresh media without Pen/Strep was added back to

each T225 and flasks were incubated an additional 3 days. On day 17 or 18, supernatants were collected as before, and combined with the previous supernatants. Supernatants were stored at -20°C and used as the source of M-CSF for bone marrow-derived macrophage preparation and culture.

Bone Marrow-Derived Macrophage Culture

BMM growth media was prepared using high glucose DMEM (Thermo Fisher Scientific) with 20% Fetal Bovine Serum (Seradigm), 1% L-glutamine (Corning), 1% Sodium pyruvate (Corning), 14mM 2-Mercaptoethanol (Gibco), and 10% 3T3 cell supernatant (from M-CSF-producing 3T3 cells). Macrophages were prepared from the femurs of C57BL/6J mice. Ripk3$^{-/-}$ femurs were generously provided by Anita Sil. Femurs were isolated, sterilized with 70% ethanol, and crushed with a mortar and pestle in BMM growth media. Cells were strained through a 70µM filter and distributed into ten 150-mm non-TC dishes in 30mL BMM culture medium. An additional 30mL BMM culture medium was added at day 3. After cells were incubated for a total of seven days at 37°C with 5% $C0_2$, cells were harvested and frozen at -80°C in BMM culture medium with 10% Fetal Bovine Serum and 10% DMSO (Sigma) added.

Blood Agar Media

Blood agar media was prepared using the following recipe for 1L of media: 5g yeast extract, 10g tryptone (BTS), 10g NaCl (Fisher), 10.48g MOPS (Sigma), 1g activated charcoal, 5g SuperPure Agar (BTS), 957.5mL MilliQ water (Millipore), and 10N NaOH to bring pH to 7.3. Media was autoclaved and cooled prior to addition of 12.5mL 304g/L glucose-1-phosphate (Sigma) and 25mL defibrinated Sheep's blood (Hemostat).

Hemolysis in BMMs

A 60mm non-TC dish with 15 12mm glass coverslips was seeded with 3 x 10^6 BMMs. The following day, the cells were infected with 2 x 10^5 CFU *hly*^{fl} for 30 minutes. Then, cells were washed with PBS and BMM media with 50µg/mL gentamicin was added. At each timepoint, coverslips were removed from the dish and placed in water to lyse the cells. Bacteria were plated on blood-agar media. Plates were incubated overnight at 37°C and then transferred to 4°C until halos surrounding hemolytic colonies were clear. Hemolytic and ahemolytic colonies were enumerated.

Hemolysis in Mice

C57BL/6J (Jackson Labs) mice were infected with 1 x 10^5 CFU of *hly*^{fl} *L. monocytogenes.* At 0, 1, 2, 3, 5, 8, and 24 hours post infection mice were euthanized (3 mice per timepoint) and spleens and livers were harvested, homogenized, and plated on blood-agar media. Plates were incubated overnight at 37°C and then transferred to 4°C until halos surrounding hemolytic colonies were clear. Hemolytic and ahemolytic colonies were enumerated.

Lactate Dehydrogenase (LDH) Assay

BMMs were seeded into a 24-well plate with 5 x 10^5 BMMs/well. The following day, cells were infected with 2x10^6 CFU L. monocytogenes for 30 minutes. Then, cells were washed with PBS and BMM media containing 5% FBS and 50µg/mL gentamicin was added to wells. 24 hours post-infection LDH Assay was performed as previously described (131).

Intracellular growth curves

3×10^6 BMMs were plated in 60 mm non-TC-treated Petri dishes with 15 12mm glass coverslips in each dish. The following day, two dishes per strain were infected with 5×10^5 CFU (MOI=0.17) and intracellular growth curves were performed as described previously (96).

Plaque assay

Six-well plates were seeded with 1.2×10^6 L2 cells per well. The plaque assay was performed as previously described (149).

Cell Spreading Assay

BMMs were seeded into a 24-well plate with glass coverslips in each well, at 3.5×10^5 cells/well. Wells were infected with bacteria. 30 minutes post infection, cells were washed with PBS and BMM media containing 50µg/mL gentamicin was added. At five hours post-infection, coverslips were removed and cells were fixed with 100% methanol and stained using Diff-Quik stain. Coverslips were mounted onto glass slides using Permount (Fisher Chemical). Light microscopy was performed using a BZ-X700 microscope (KEYENCE) and a 60x objective lens. Cell spread index was calculated by counting the number of cells in an infectious focus containing 5 or more bacteria and subtracting one cell so that the cell spread index represents the number of cells that were spread to from the initially infected cell. Analysis was performed blindly. In total, 60 *hly*^{fl} and 67 WT infectious foci were analyzed.

Animal Use Ethics Statement

All animal work was done in strict accordance with university regulations. Protocols were reviewed and approved by the Animal Care and Use Committee at the University of California, Berkeley AUP-2016-05-8811.

Virulence in Mice

Eight-week-old female CD-1 mice (Charles River) were infected intravenously with 1×10^5 CFU in 200µL PBS. Forty-eight hours post-infection the mice were euthanized and spleens and livers were harvested, homogenized in 0.1% IGEPAL CA-630 (Sigma) in water, and plated on LB Agar with 200µg/mL streptomycin for enumeration of bacterial burdens.

Vaccination of Mice

Eight-to-ten-week-old female C57BL/6J mice (Jackson Laboratories) were vaccinated by intravenous injection of *L. monocytogenes* in 200µL PBS. Four-weeks post vaccination, mice were challenged with 5×10^4 CFU WT *L. monocytogenes* injected intravenously in 200µL PBS. Three-days post challenge, mice were euthanized, and spleens and livers were harvested, homogenized in 0.1% IGEPAL CA-630 (Sigma), and plated on LB Agar with 200µg/mL streptomycin for enumeration of bacterial burdens. Mice treated with αIL-10R antibody (Clone 1B1.3A, Bio X Cell) were injected with 250µg of antibody in 100µL PBS two hours prior to vaccination.

Statistical Analysis

Data were analyzed using GraphPad Prism 8. For mouse CFU experiments, data was log-transformed prior to performing statistical analysis. * indicates $P < 0.05$; ** indicates $P < 0.01$, *** indicates $P < 0.001$, **** indicates $P < 0.0001$; ns indicates no statistical significance.

Table 2. Strains

Strain Name	Strain Background	Plasmid 1	Plasmid 2	Strain Number	Reference
hly^{fl}	Δ*hly*	pPL1-*hly*^{fl}	pPL2e-*actA-cre*	DP-L6648	(114)
Δ*hly* + *hly*^{fl} (complement)	Δ*hly*	pPL1-*hly*^{fl}	-	DP-L6647	(114)
WT	WT 10403S (DP-L6234; DP-L4056 phage-cured)	pPL1-*tetL*^{fl}	-	DP-L6896	This study
Δ*hly*	Δ*hly* (DP-L6236; DP-L4027 phage-cured)	pPL1-*tetL*^{fl}	-	DP-L6897	This study
Δ*actA*	Δ*actA* (DP-L6235; DP-L4029 phage-cured)	pPL1-*tetL*^{fl}	-	DP-L6894	This study
Δ*actA hly*^{fl}	Δ*actA*Δ*hly* phage-cured (DP-L6237)	pPL1-*hly*^{fl}	pPL2e-*actA-cre*	DP-L6883	This study
pPL1-*hly*^{fl}	SM10			DP-E6869	This study
pPL1-*tetL*^{fl}	SM10			DP-E6892	This study
pPL2e-*actA-cre*	SM10			DP-E6233	(146)

Chapter 3
Characterization of TLR2 and endosomal TLR-mediated secretion of IL-10 and immune suppression in response to phagosome-confined *Listeria monocytogenes*

3.1 Summary of Results

Listeria monocytogenes is a facultative intracellular pathogen that induces a robust adaptive immune response in mice characterized by the induction of antigen specific CD8+ T-cells. However, *L. monocytogenes* mutants unable to escape phagosomes fail to induce a robust adaptive immune response and suppress the response to wildtype bacteria when co-administered. The capacity to suppress immunity can be reversed by blocking IL-10. In this study, we sought to understand the host cell signaling pathways that lead to secretion of IL-10 in response to phagosome-confined *L. monocytogenes* (Δ*hly*). We conducted a transposon screen to identify Δ*hly L. monocytogenes* mutants that induced significantly more or less IL-10 secretion in bone marrow-derived macrophages (BMMs). A transposon insertion in *lgt*, which encodes phosphatidylglycerol-prolipoprotein diacylglyceryl transferase and is essential for the formation of lipoproteins, significantly reduced IL-10 secretion from BMMs. Mutants with transposon insertions in *pgdA* and *oatA*, which encode peptidoglycan N-acetylglucosamine deacetylase and O-acetyltransferase and are sensitive to lysozyme, induced enhanced IL-10 secretion. In BMMs, secretion of IL-10 in response to infection with the parental strain was mostly TLR2-dependent, while IL-10 induced by strains engineered to lyse was dependent on TLR2 and Unc93b1. In mice, nucleic acid-sensing TLRs primarily mediated the IL-10 response to vacuole-confined *L. monocytogenes.* Co-administration of Δ*hly* and Δ*actA* resulted in suppressed immunity in WT mice, but not in mice with mutations in Unc93b1. These data revealed that secretion of IL-10 in response to *L. monocytogenes* infection *in vitro* is mostly TLR2-dependent and immune suppression by phagosome-confined bacteria *in vivo* is mostly dependent on endosomal TLRs.

3.2 Introduction

Listeria monocytogenes is a Gram-positive facultative intracellular pathogen that has been widely used as a model to study host immune responses. In mice, primary infection with *L. monocytogenes* induces the generation of adaptive immune responses that are protective against subsequent infections and are mediated by CD8+ T cells (150,151). Many innate immune factors contribute to control of primary *L. monocytogenes* infection and development of functional $CD8^+$ effector and memory cells (152–155).

It has been posited that intracytosolic growth of *L. monocytogenes* is a prerequisite for the induction of T cell-mediated immunity because Δ*hly L. monocytogenes*, which does not produce the virulence factor Listeriolysin O and cannot escape phagocytic vacuoles, fails to induce robust protective immunity (156,157). However, in 2009 Bahjat *et al.* provided evidence that Δ*hly L. monocytogenes* fails to induce robust protective immunity because it induces secretion of IL-10 early during infection. When IL-10 signaling was inhibited by administration of anti-IL-10 receptor blocking antibody, Δ*hly L. monocytogenes* induced enhanced protective immunity (130). Thus, strains that escape phagocytic vacuoles and grow in the cytosol induce generation of protective immunity in part because they avoid inducing IL-10 secretion.

IL-10 is an anti-inflammatory cytokine that acts on many cell types to downregulate inflammation and recruitment of immune cells, ideally limiting immunopathology during the resolution of an immune response (158). Infection of wildtype (WT) mice with *L. monocytogenes* results in high levels of serum IL-10 3-4 days post infection (159,160). Mice lacking IL-10 clear *L. monocytogenes* faster than wildtype mice, indicating that IL-10 partially suppresses the primary innate immune response to *L. monocytogenes* (161,162). Importantly, though IL-10 is expressed following primary WT *L. monocytogenes* infection, WT infection results in the generation of a robust protective immune response.

IL-10 is secreted by many cell types, including macrophages, dendritic cells (DCs), neutrophils, NK cells, T-regulatory cells and B cells (158). The IL-10 present in serum 3 to 4 days after wildtype *L. monocytogenes* infection of mice is mostly derived from NK cells (159). However, Δ*hly L. monocytogenes* induces IL-10 secretion that is detectable four hours after infection, and likely comes from macrophages or dendritic cells, which are the first cells infected by *L. monocytogenes* in the spleen (130,137,138). In macrophages, IL-10 expression can be triggered by activation of the pattern recognition receptors TLR2, TLR3, TLR4, TLR9 (158,163). Macrophages secrete high levels of IL-10 in response to the TLR9 agonist CpG, while myeloid DCs secrete much less (163). Myeloid DCs can secrete IL-10 upon activation of TLR2, TLR4, TLR9, and also the C-type lectins DC-SIGN and Dectin-1 (158,164–166). Thus, there are diverse pathways leading to IL-10 secretion. The timing of IL-10 secretion and cell types that respond can impact the generation of adaptive immune responses (167).

The bacterial components and signaling pathways that lead to induction of IL-10 following infection with Δ*hly L. monocytogenes* have not been experimentally addressed. In this study, we conducted a genetic screen to identify the components of vacuole-confined Δ*hly L. monocytogenes* that induce IL-10 secretion from murine bone marrow-derived macrophages. We investigated the host signaling pathways that lead to recognition of Δ*hly L. monocytogenes* and secretion of IL-10 in macrophages and mice, and how these host signaling pathways affect vaccination and immune suppression.

3.3 Results

3.3.1 Genetic screen to identify *L. monocytogenes* mutants that induce enhanced or diminished levels of IL-10

The goal of this study was to identify *L. monocytogenes* determinants that contribute to induction of IL-10 secretion from BMMs. We screened a library of Δ*hly* transposon mutants for their ability to induce IL-10 secretion from BMMs. The transposon library was generated in a flagellin-negative Δ*hly* background (Δ*hly*Δ*fla*) to eliminate the identification of false low-IL-10 mutants resulting from mutations in flagellar components that reduce infection efficiency. Previous work demonstrated that infection of mice with Δ*hly* is immunosuppressive and IL-10 levels are increased in the serum four hours post-infection (130), while infection with WT leads to IL-10 that peaks 3-4 days post-infection (160,161). To determine whether significant IL-10 is secreted by *L. monocytogenes*-infected BMMs four hours post-infection, BMMs were infected with Δ*hly* and Δ*hly*Δ*fla L. monocytogenes*, and IL-10 was measured from cell supernatants. BMMs infected with both Δ*hly* and Δ*hly*Δ*fla L. monocytogenes* secreted significantly more IL-10 than uninfected BMMs four hours post-infection (Fig 3.1A). As *L. monocytogenes* infection leads to significant expression of IL-10 in both BMMs and mice four hours post-infection, we analyzed cell supernatants for IL-10 levels four hours post-infection in our screen.

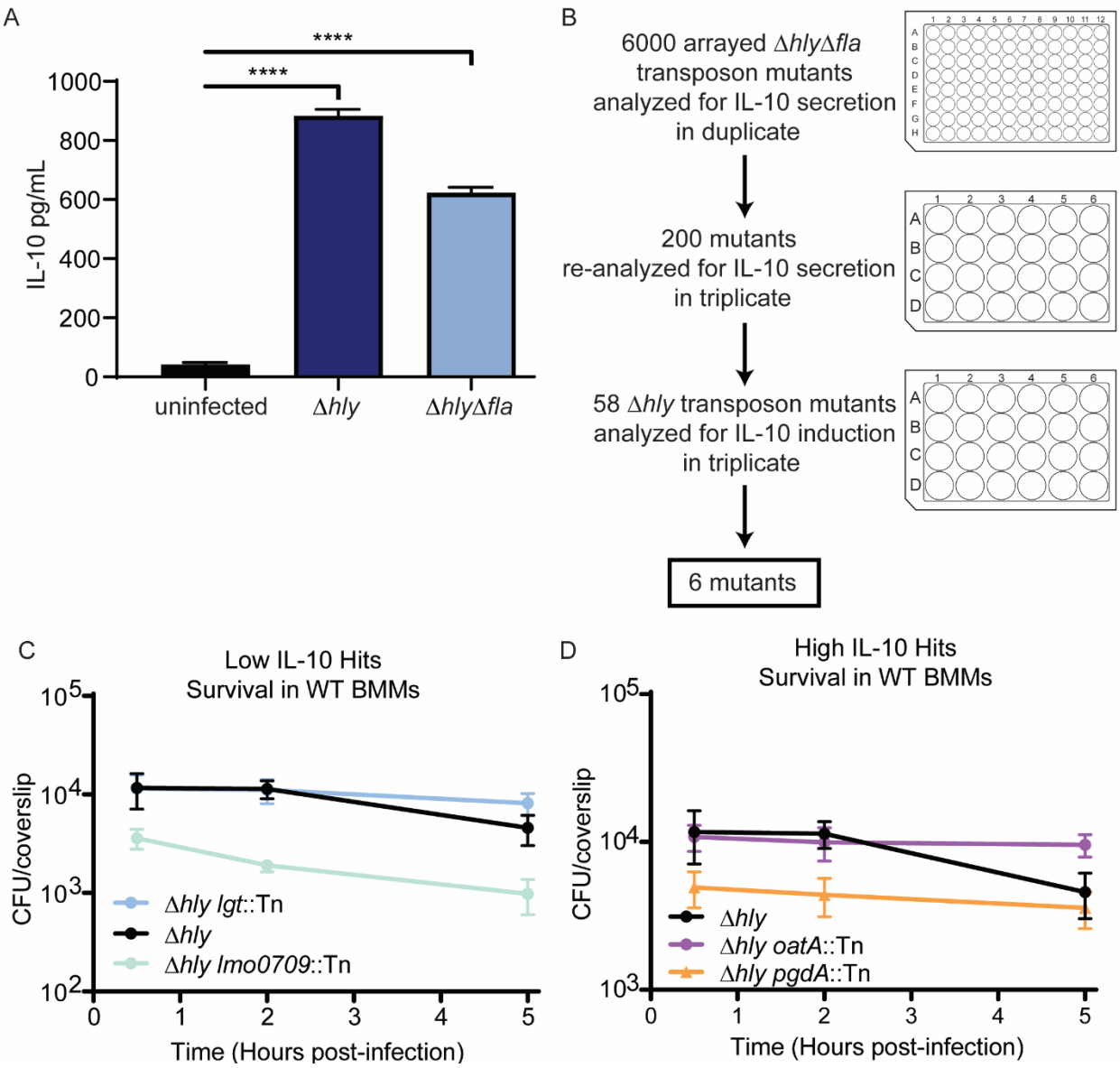


Figure 3.1. Screen for Δ*hly L. monocytogenes* mutants that induce enhanced or diminished IL-10.

(A) BMMs were infected with *L. monocytogenes* lacking LLO (Δ*hly*) or lacking LLO and flagellin (Δ*hly*Δ*fla*) for four hours. Cell supernatant was collected and IL-10 was measured by ELISA. Data shown is representative of two independent experiments. Mean and SD are shown. n=3. Data analyzed using Holm-Sidak's multiple comparisons test. (B) Schematic of screen. 6000 Δ*hly*Δ*fla* transposon mutants were analyzed for enhanced or diminished IL-10 expression in duplicate in a 96-well plate format. 200 mutants were selected for secondary screening, and were analyzed for IL-10 in triplicate in a 24-well plate format. The mutations from 58 mutants were transduced into a Δ*hly* background. Six mutations that resulted in enhanced or diminished IL-10 secretion were identified. (C and D) Survival of transposon mutants was quantified in BMMs. Mean and SEM are shown. Data is pooled from three coverslips per experiment for three independent experiments.

BMMs were infected in duplicate with 6000 gridded Δ*hly*Δ*fla* transposon mutants in 96-well plates (Screen Scheme in Fig 3.1B). 200 mutants that induced enhanced or diminished IL-10 levels compared to Δ*hly*Δ*fla* were selected for secondary screening. For secondary screening, optical density of bacterial cultures was adjusted to eliminate differences in IL-10 secretion due to growth differences in broth. 59 of the 200 mutants induced significantly enhanced or diminished IL-10 levels compared to Δ*hly*Δ*fla* in a 24-well format (Supplemental Table 3.1). The transposons in 58 mutants were phage-transduced from the Δ*hly*Δ*fla* background into a Δ*hly* background. In the Δ*hly* background, only two mutants induced less IL-10 secretion and four mutants induced increased IL-10 secretion (Table 3.1).

Diminished IL-10	**Gene Annotation**	**IL-10 (% Δ*hly*)**	**Strain Number**
Δ*lgt*	*lgt*	30	DP-L7003
Lmo2482::Tn	*lgt*	27	DP-L7111
Lmo0709::Tn	hypothetical protein	41	DP-L7080
Enhanced IL-10	**Gene Annotation**	**IL-10 (% Δ*hly*)**	**Strain Number**
Δ*pgdA*Δ*oatA*	*pgdA/oatA*	263	DP-L7004
Lmo0415::Tn	*pgdA*	177	DP-L7074
Lmo2529::Tn	ATP synthase F0F1 subunit beta	158	DP-L7112
Lmo2634::Tn	*ecfT*	140	DP-L7116
Lmo1291::Tn	*oatA*	139	DP-L7089

Table 3.1. IL-10 secretion four hours post-infection of BMMs with transposon mutants identified by genetic screen and deletion mutants.

Notably, a mutant with a transposon insertion in phosphatidylglycerol-prolipoprotein diacylglyceryl transferase (*lgt*), induced nearly no IL-10 secretion, though it entered cells and survived in cells similar to Δ*hly* (Fig 3.1C). Lgt catalyzes the transfer of a lipid moiety from phosphatidylglycerol onto a cysteine residue of prolipoproteins (168–170). The resulting lipoproteins are well-known TLR2 agonists. Of the two mutants that induced reduced IL-10 secretion, we focused on understanding the contribution of *lgt* to the induction of IL-10 secretion from BMMs because the other mutant, which had a transposon insertion in lmo0709, had reduced infection capability (Fig 3.1C).

To confirm the role of *lgt* in the reduced IL-10 phenotype, an in-frame deletion of *lgt* was generated in a Δ*hly* background. Deletion of *lgt* did not affect infection efficiency or survival in BMMs (Fig 3.2B). *Lgt*-deletion mutants induced significantly reduced IL-10 secretion from WT BMMs compared to Δ*hly* and Δ*hly*Δ*fla*. IL-10 levels were restored when *lgt* was complemented in Δ*hly* and Δ*hly*Δ*fla L. monocytogenes* under control of the constitutively active pHyper promoter (Figure 3.2A). These data indicated that Δ*hly L. monocytogenes* primarily induces IL-10 secretion in a lipoprotein- and TLR2-dependent manner.

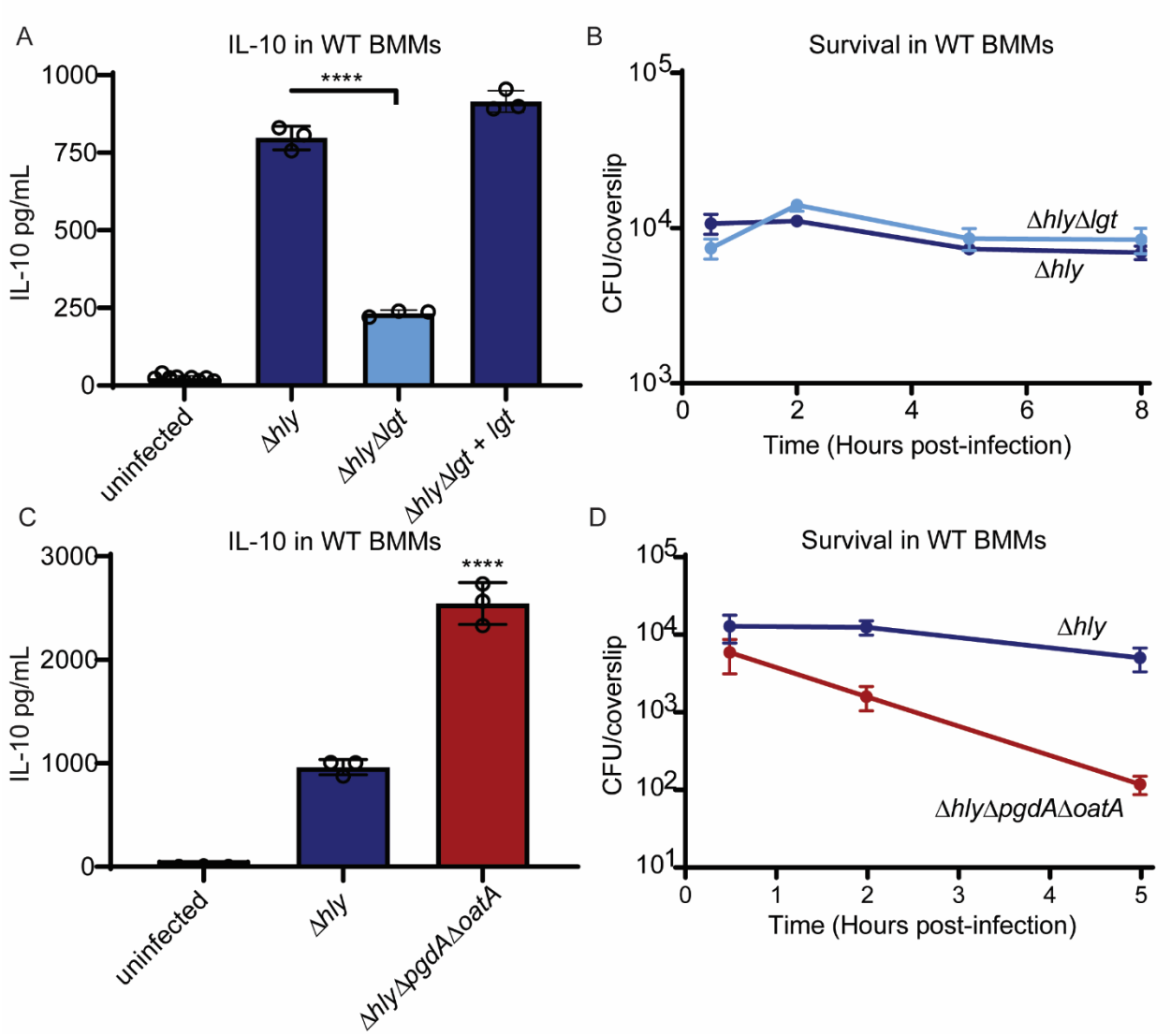


Figure 3.2. Δ*hly*-induced IL-10 secretion is largely *lgt*-dependent and lysis leads to increased IL-10 secretion.

(A) IL-10 secretion from BMMs four hours-post infection. Mean and SD are shown. Data is representative of two independent experiments. Data analyzed using Holm-Sidak's multiple comparison's test. (B) Survival of *lgt* deletion mutant in BMMs. Data is representative of two independent experiments. Mean and SEM are shown. (C) IL-10 secretion from BMMs four hours-post infection. Mean and SD are shown. Data is representative of two independent experiments. Data analyzed using Holm-Sidak's multiple comparison's test. (B) Survival of Δ*hly*Δ*pgdA*Δ*oatA* deletion mutant in BMMs. Data is pooled from three coverslips per experiment for three independent experiments. Mean and SEM are shown.

Of the mutants that induced increased IL-10 secretion, mutations in two of the genes, *pgdA* and *oatA*, have previously been shown to confer sensitivity to lysozyme (171). We hypothesized that these mutants had increased bacterial lysis within host phagosomes, leading to greater activation of endosomal nucleic acid-sensing TLRs. Though the mutations did not contribute to a noticeable survival defect individually, a strain lacking both *pgdA* and *oatA* (Δ*hly*Δ*pgdA*Δ*oatA*) had a significant survival defect in BMMs (Fig 3.2D), indicative of bacterial death in the vacuole. We hypothesized that lysis of bacteria in the phagocytic vacuole could result in release of bacterial nucleic acids and activation of nucleic acid-sensing TLRs.

3.3.2 IL-10 secretion in BMMs

To analyze the role of TLR2 in secretion of IL-10, we infected macrophages lacking TLR2. While infection with Δ*hly* induced significant IL-10 secretion from WT BMMs, Δ*hly* induced almost no IL-10 secretion in TLR2$^{-/-}$ BMMs (Fig 3.3A), suggesting that bacterial lipoproteins are the major activators of IL-10 secretion in response to infection with Δ*hly*. In contrast, Δ*hly*Δ*pgdA*Δ*oatA* induced significant levels of IL-10 secretion in TLR2$^{-/-}$ BMMs (Fig 3.3B), suggesting that additional bacterial lysis can stimulate a second pathway of IL-10 induction. To determine whether this second pathway was the endosomal nucleic-acid sensing TLRs, we infected BMMs with a mutation in the endosomal TLR trafficking protein Unc93b1, which is defective in endosomal TLR signaling (172). Δ*hly* induced similar amounts of IL-10 in WT and Unc93b1$^{3d/3d}$ BMMs. (Fig 3.3A). However, Δ*hly*Δ*pgdA*Δ*oatA* induced significantly less IL-10 secretion in Unc93b1$^{3d/3d}$ BMMs (Fig 3B).

To confirm that IL-10 secretion in response to vacuole-confined *L. monocytogenes* resulted from signaling through TLR2 and endosomal TLRs, we infected BMMs with mutations in both pathways (TLR2$^{-/-}$TLR5$^{-/-}$Unc93b1$^{3d/3d}$). These macrophages secreted IL-10 in response to LPS, which is a TLR4 ligand, but not in response to the *L. monocytogenes* mutants that were tested (Fig.3.3C). These results confirmed that IL-10 secretion from BMMs results from signaling through TLR2 and endosomal TLRs.

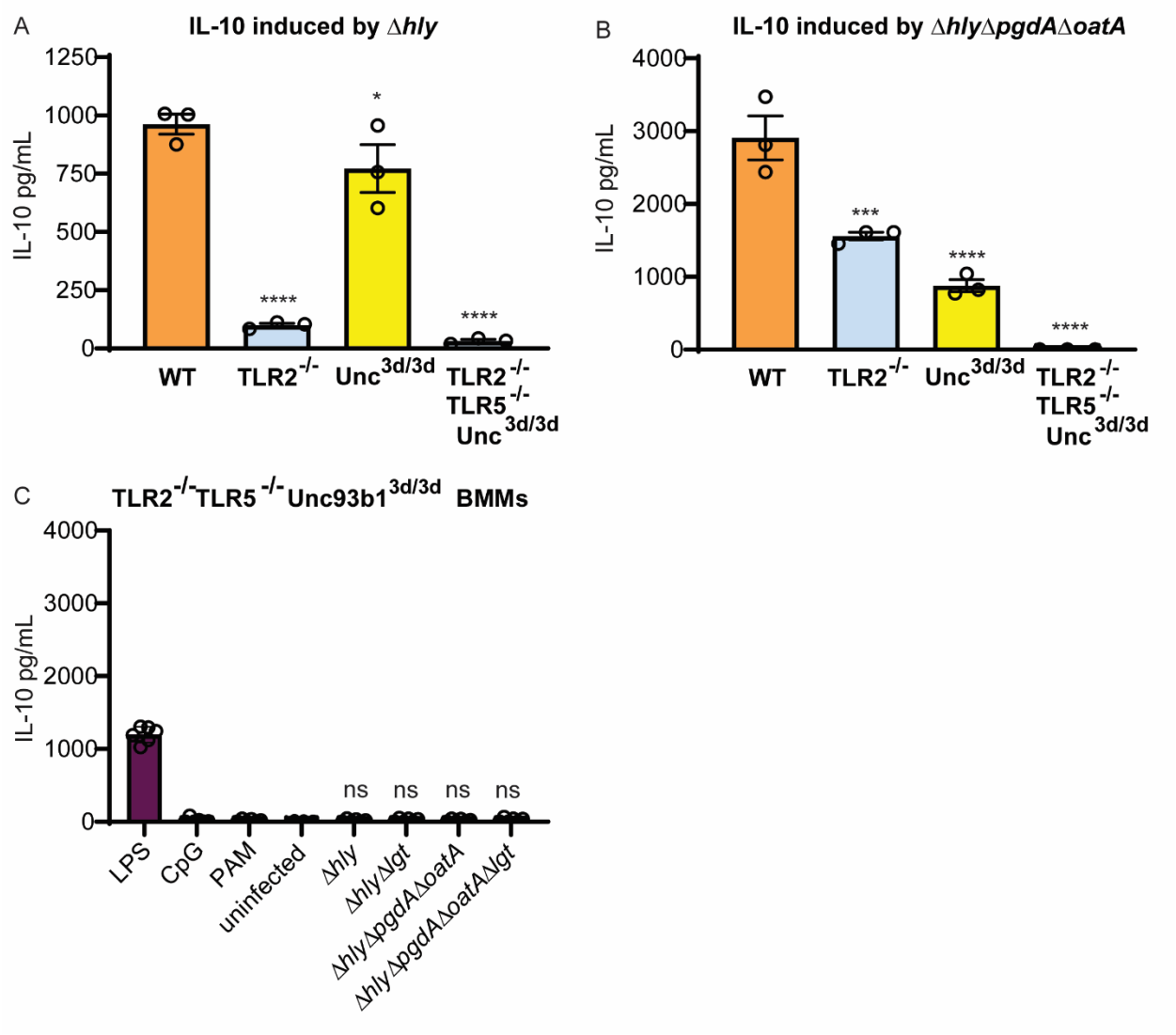


Figure 3.3 IL-10 secretion from BMMs in response to *L. monocytogenes* infection requires TLR2 and endosomal TLR signaling.

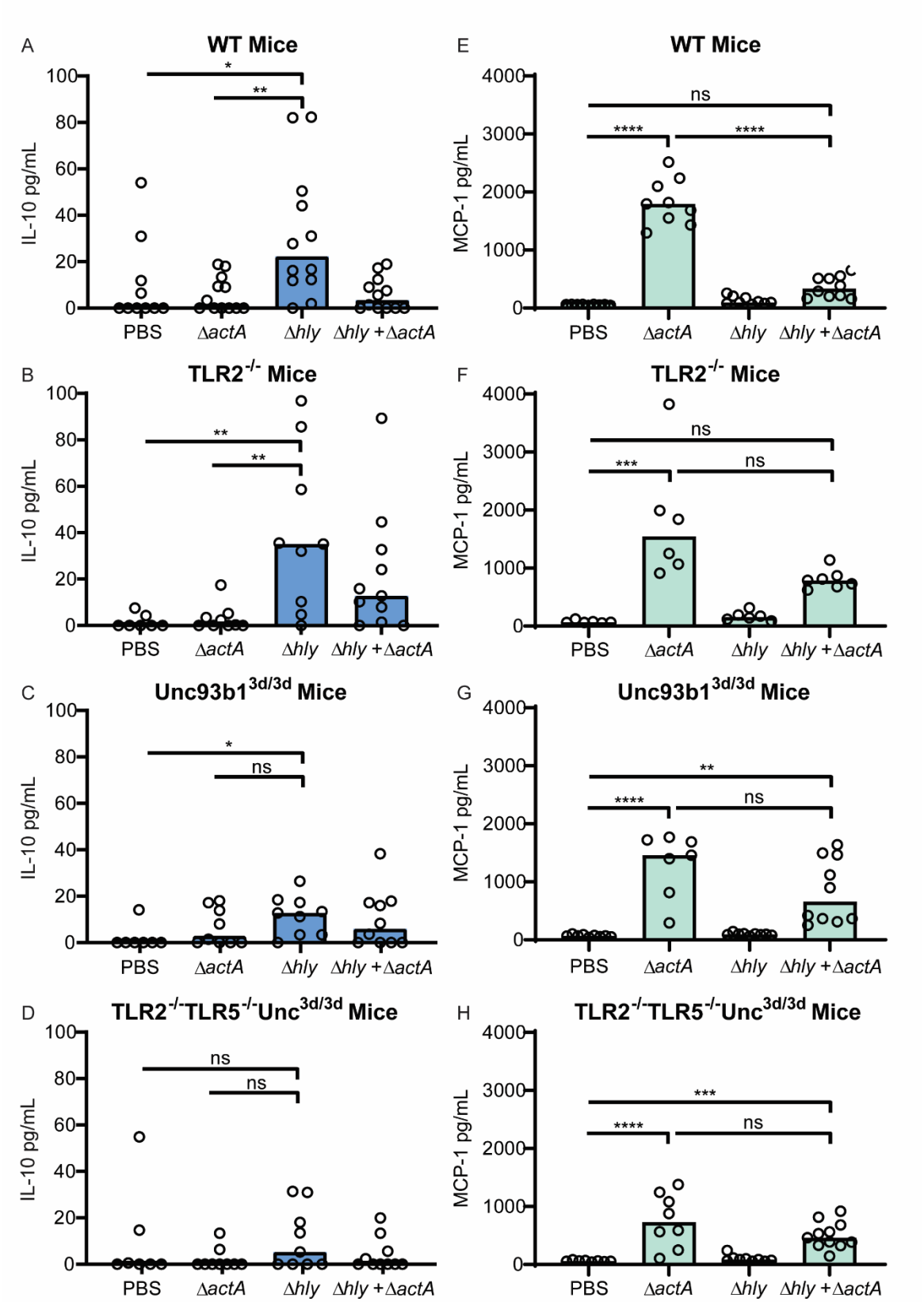
WT, TLR2$^{-/-}$, Unc93b1$^{-/-}$, and TLR2$^{-/-}$TLR5$^{-/-}$Unc93b1$^{3d/3d}$ BMMs were infected with Δ*hly L. monocytogenes* (A) or Δ*hly*Δ*pgdA*Δ*oatA L. monocytogenes* (B). Cell supernatant was harvested four hours post infection and IL-10 was measured by ELISA. Data shown is representative of two independent experiments. Means were compared to WT using Holm-Sidak's Multiple Comparisons test. (C) TLR2$^{-/-}$TLR5$^{-/-}$Unc93b1$^{3d/3d}$ BMMs were infected with the indicated strains of *L. monocytogenes*, or treated with LPS, a TLR4 agonist; CpG, a TLR9 agonist; or Pam2CSK4, a TLR2 agonist. Data shown is pooled from two independent experiments. Data analyzed using Holm-Sidak's Multiple Comparisons test.

3.3.3 Cytokine secretion in mice

To determine whether secretion of IL-10 in mice was dependent on TLR2 and/or endosomal TLRs, mice were infected intravenously and serum IL-10 four hours post-infection was quantified (Fig 3.4A-D). Infection of WT mice with Δ*hly* resulted in significant levels of IL-10. Unlike in BMMs, Δ*hly* also induced significant amounts of IL-10 in TLR2$^{-/-}$ mice (Fig 3.4B), suggesting that bacterial lipoproteins were not the dominant IL-10-inducing molecules in mice. In contrast, IL-10 secretion in Unc93b1$^{3d/3d}$ or TLR2$^{-/-}$TLR5$^{-/-}$Unc93b1$^{3d/3d}$ mice was almost zero (Fig 3.4C and 3.4D). Together, these results indicate that endosomal TLRs are the primary IL-10 signaling pathways that respond to Δ*hly* infection in mice.

One function of IL-10 signaling is to limit proinflammatory cytokine secretion. To investigate the relationship between TLRs and proinflammatory cytokine signaling, serum levels of MCP-1 were quantified 24 hours post-infection (Fig 3.4E-H). In WT mice, Δ*hly* did not induce secretion of MCP-1, while Δ*actA*, a strain of *L. monocytogenes* that escapes the phagocytic vacuole and replicates in the host cell cytosol, induced significant amounts of MCP-1 (Fig 3.4E). Likewise, Δ*actA*, but not Δ*hly*, induced significant MCP-1 secretion in TLR2$^{-/-}$ mice (Fig 3.4F-H). Because early IL-10 expression is thought to limit later expression of proinflammatory cytokines, we hypothesized that Δ*hly* would induce proinflammatory cytokines in Unc93b1$^{3d/3d}$ and TLR2$^{-/-}$TLR5$^{-/-}$Unc93b1$^{3d/3d}$ mice. However, MCP-1 was not induced in response to Δ*hly* background strains in Unc93b1$^{3d/3d}$ and TLR2$^{-/-}$TLR5$^{-/-}$Unc93b1$^{3d/3d}$ mice (Fig 3.4G and 3.4H). These results indicated that endosomal TLR signaling may be required for Δ*hly* to induce a proinflammatory cytokine response.

Vaccination of mice with Δ*actA* has previously been shown to result in high levels of proinflammatory cytokines, whereas vaccination of mice with Δ*hly* induced high levels of IL-10 and low levels of proinflammatory cytokines in WT mice. Strikingly, co-administration of Δ*actA* and Δ*hly* resulted in high levels of IL-10 and low levels of proinflammatory cytokines in WT mice (130), suggesting that Δ*hly* suppresses the proinflammatory responses usually induced by Δ*actA*. We analyzed the levels of MCP-1 in serum as a measure of the proinflammatory cytokine response. It was previously reported that Δ*actA* induced significant amounts of MCP-1 in WT mice, but that co-administration of Δ*actA* with 1000-fold more Δ*hly* CFU resulted in reduced expression of MCP-1 (130). Co-administration of Δ*actA* and Δ*hly* did not result in significant MCP-1 secretion in WT mice, consistent with previous findings. Co-administration of Δ*actA* and Δ*hly* in TLR2$^{-/-}$ mice also did not result in significant MCP-1 secretion, suggesting that immunosuppression by Δ*hly* is not mediated by TLR2 signaling (Fig 3.4E and 3.4F). However, co-administration of Δ*actA* and Δ*hly* resulted in significant secretion of MCP-1 in Unc93b1$^{3d/3d}$ and TLR2$^{-/-}$TLR5$^{-/-}$Unc93b1$^{3d/3d}$ mice (Fig 3.4G and 3.4H). These results indicated that suppression of proinflammatory cytokines by Δ*hly* is mediated by Unc93b1-dependent TLRs.


A
WT Mice
IL-10 pg/mL
100
80
60
40
20
0
*
**
PBS
ΔactA
Δhly
Δhly +ΔactA
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WT Mice
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3000
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TLR2-/- Mice
IL-10 pg/mL
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Figure 3.4. IL-10 secretion in mice is dependent on endosomal TLR signaling.

Mice were infected with 10^8 CFU of Δ*hly*, 10^5 CFU of Δ*actA*, or a combination of 10^8 CFU of Δ*hly* and 10^5 CFU of Δ*actA*. WT C57BL/6J mice (A and E), TLR2$^{-/-}$ (B and F), Unc93b1$^{3d/3d}$ (C and G), and TLR2$^{-/-}$TLR5$^{-/-}$Unc93b1$^{3d/3d}$ (D and H) were infected. Serum measurements of IL-10 four hours post-infection (A-D) and MCP-1 twenty-four hours post-infection (E-H). Data is pooled from two to four independent experiments. Bar represents the median. Data analyzed using Holm-Sidak's Multiple Comparisons test.

3.3.4 Suppression of adaptive immunity

To investigate the relationship between IL-10 signaling pathways and suppression of adaptive immunity, co-vaccination experiments were performed in which Δ*actA* and 1000-fold more Δ*hly* CFU were injected simultaneously as described in (130) (Fig 3.5). In WT mice, vaccination with Δ*actA* leads to the induction of robust adaptive immunity that can nearly clear a subsequent lethal dose of WT *L. monocytogenes.* However, the immunity induced by Δ*actA* is suppressed two-logs by co-administration of Δ*hly* (Fig 3.5A). This suppression was previously shown to depend on IL-10 (157). We hypothesized that Δ*hly* would suppress immunity in WT and TLR2$^{-/-}$ mice, in which we observed significant secretion of IL-10 and low levels of MCP-1, but not in Unc93b1$^{3d/3d}$ or TLR2$^{-/-}$TLR5$^{-/-}$Unc93b1$^{3d/3d}$ mice, in which we observed no IL-10 and high levels of MCP-1. That was indeed the case, as we observed 2-logs of immune suppression in WT and TLR2$^{-/-}$ mice (Fig 3.5A and 3.5B). However, in Unc93b1$^{3d/3d}$ or TLR2$^{-/-}$TLR5$^{-/-}$ Unc93b1$^{3d/3d}$ mice co-administration of Δ*actA* with Δ*hly* did not reduce protective immunity (Fig 3.5C and 3.5D). Thus, vacuole-confined *L. monocytogenes* induce IL-10 in mice primarily through endosomal TLRs which leads to suppression of adaptive immunity.

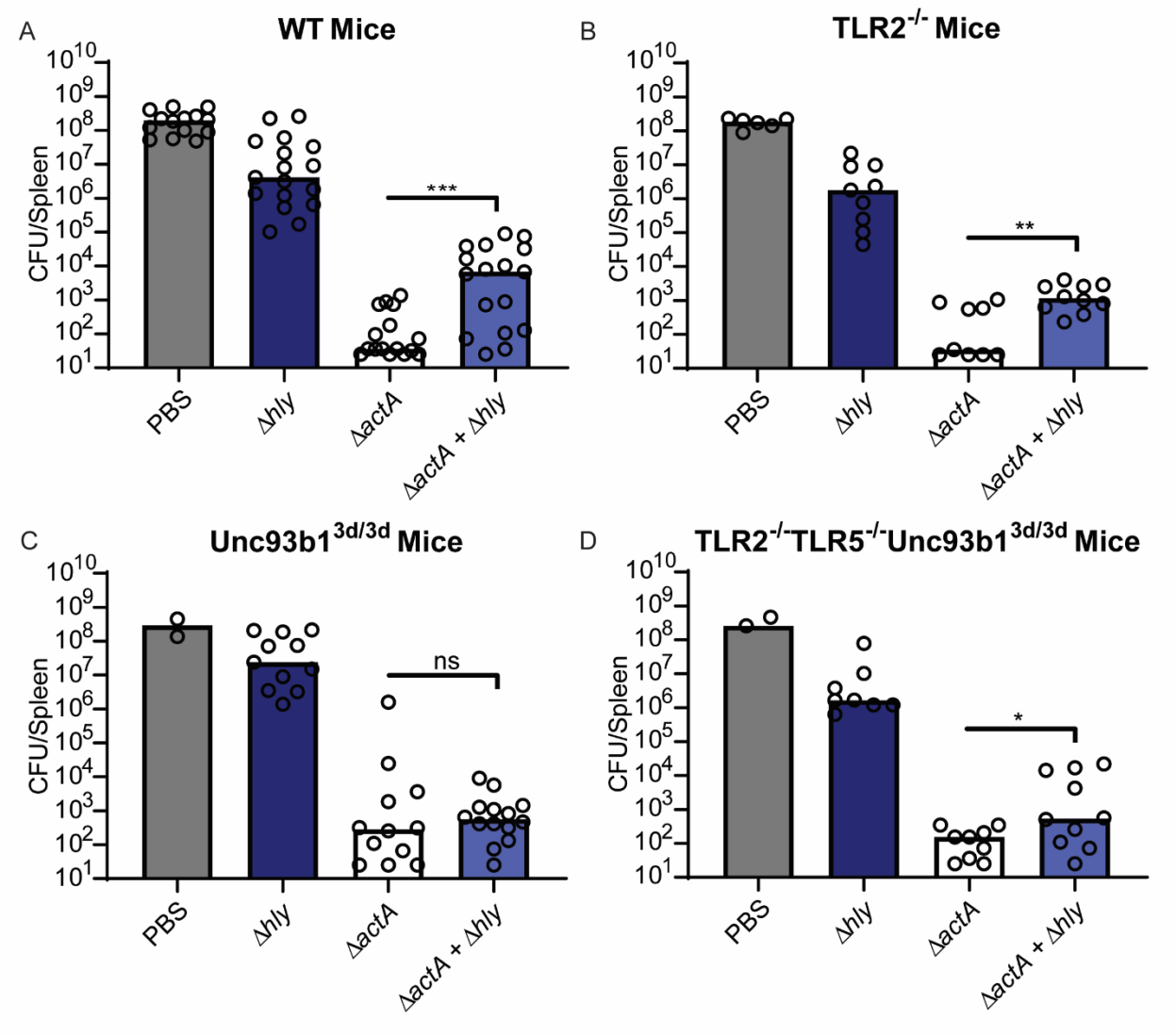


Figure 3.5. Immune suppression is primarily mediated by endosomal TLR signaling.

Mice were infected with 10^8 CFU of Δ*hly*, 10^5 CFU of Δ*actA*, or a combination of 10^8 CFU of Δ*hly* and 10^5 CFU of Δ*actA*. WT C57BL/6J mice (A), TLR2$^{-/-}$ (B), Unc93b1$^{3d/3d}$ (C), and TLR2$^{-/-}$TLR5$^{-/-}$Unc93b1$^{3d/3d}$ (D) were infected. Four-to-eight weeks post-vaccination, mice were challenged with 5x10^5 WT *L. monocytogenes.* CFU from the spleen were enumerated three days post-challenge. Data is pooled from two to five independent experiments. Bar represents the median. Data analyzed using an unpaired t test.

3.4 Discussion

The results of this study show that the secretion of IL-10 by BMMs is largely mediated by TLR2-detection of bacterial lipoproteins. However, analysis of mutants that induced enhanced IL-10 revealed a second pathway that was dependent on bacterial lysis triggering nucleic-acid sensing TLRs in the phagosome. In mice, nucleic acid-sensing TLRs were the dominant trigger of IL-10 secretion.

The differences in TLR contributions to IL-10 expression in BMMs compared to mice likely reflects the differing abilities of macrophages to kill and degrade *L. monocytogenes.* In BMMs, which are differentiated and cultured *ex vivo*, Δ*hly L. monocytogenes* cannot grow but are not efficiently killed (Fig 3.2B). In contrast, peritoneal macrophages taken directly from mice kill vacuole-confined bacteria (173). In the absence of bacterial killing, bacterial lipoproteins are the most abundant TLR stimulus. In mice, we surmise that increased bacterial killing leads to release of nucleic acids and lipoproteins that are sensed by endosomal TLRs and TLR2, respectively. The results of our analysis of BMMs indicates that TLR2 and Unc93b1-dependent TLRs represent two independent pathways that lead to IL-10 expression. However, our data in mice indicates that TLR2-signaling does not contribute to IL-10 expression to the same extent as endosomal TLRs. Unc93b1$^{3d/3d}$ mice retain signaling through TLR2, but did not express significant IL-10 following infection with Δ*hly* (Fig 3.4C). In contrast, TLR2-deficient mice expressed significant IL-10 following infection with Δ*hly* (Fig 3.4B). This IL-10 was sufficient to inhibit the generation of protective immunity by Δ*actA*, indicating that IL-10 downstream of endosomal TLRs is the major mediator of immune suppression.

It was shown previously that administration of anti-IL-10 receptor blocking antibody restored the protective potential of Δ*hly*. However, in this study we observed that, in mice with a defect in endosomal TLR signaling, vaccination with Δ*hly L. monocytogenes* did not induce IL-10 secretion but still did not lead to the generation of a protective immune response. A proinflammatory response was also not induced in Unc93b1 mutant mice following vaccination with Δ*hly*, which may explain why a protective immune response was not generated. The differences in immunity following vaccination of WT mice with Δ*hly* and anti-IL10R blocking antibody and vaccination of Unc93b1 mutant mice with Δ*hly* may reflect the fact that anti-IL10R blocking antibody only blocks IL-10 signaling, while mice with defects in endosomal TLR signaling may not be able to generate a proinflammatory response to Δ*hly* and are thus inhibited in generating protective immunity.

Although the results of our study suggest that recognition of bacterial lipoproteins by TLR2 is not the primary reason for induction of IL-10, TLR2 is still an important host receptor to consider for the development of bacterial vaccine delivery systems. *L. monocytogenes* expresses over 30 diacylated lipoproteins that are recognized by TLR2/TLR6 dimers (174–177). Deletion of *lgt* in WT *L. monocytogenes* results in delayed secretion of proinflammatory cytokines, but only a modest virulence defect in mice (178). Thus, removing lipoproteins is a promising strategy for improving *L. monocytogenes*-based vaccines by changing the cytokine response without significantly affecting bacterial fitness. One potential method of modulating the response to lipoproteins that has not been explored in *L. monocytogenes* is the engineering of *L. monocytogenes* lipoproteins with different acylation states. Triacylated lipoproteins are recognized by TLR2/TLR1 heterodimers and elicit a different inflammatory response than diacylated lipoproteins (179). More recently, three additional classes of lipoprotein modification with differing abilities to stimulate TLR2 have been described in Gram-positive bacteria (174). It

would be interesting to investigate the contributions of differently acylated lipoproteins to the development of adaptive immunity. Modulating the expression of individual lipoproteins could also impact the immune response. It is not clear whether all *L. monocytogenes* lipoproteins contribute to TLR2 activation, or whether *L. monocytogenes* has a subset of lipoproteins that specifically activate TLR2. Though the lipid portion of lipoproteins mediates binding of lipoproteins to TLR2, the sequence of the attached peptides affects the ability of lipoproteins to differentially stimulate cytokine secretion (180). Therefore, increasing or decreasing the expression of specific lipoproteins could be a strategy for fine-tuning the proinflammatory immune response. In addition, TLR2 is traditionally considered a cell-surface-localized TLR, but there is evidence that lipoproteins can activate TLR2 signaling in the endosomal compartment and that signaling from the endosomal compartment can have different effects than from the plasma membrane (181–185). How specific lipoproteins and acylation state contribute to activation of TLR2, and how TLR2 activation on the cell surface versus in the lysosome contributes to the cytokine response is relevant for the development of bacterial vaccine vectors and warrants further study.

The results of this study indicated that, in mice, endosomal TLRs sense vacuole-confined *L. monocytogenes*, resulting in the secretion of IL-10. In our genetic screen, we identified mutants that had increased IL-10 secretion in WT, TLR2$^{-/-}$, and Unc93b1$^{3d/3d}$ macrophages (Supplemental Table 3.2), including transposon insertions in *pgdA* and *oatA*, genes for which mutations have previously been shown to confer increased lysozyme sensitivity (186). Enhanced IL-10 expression in response to *L. monocytogenes* mutants was dependent on both TLR2 and endosomal TLRs, as no mutants induced detectable IL-10 secretion in TLR2$^{-/-}$TLR5$^{-/-}$ Unc93b1$^{3d/3d}$ BMMs (Supplemental Table 3.2), suggesting that bacterial lysis releases nucleic acids and lipoproteins that are both sensed by the cell. Though we did not observe a decrease in colony forming units of Δ*hly* or the *pgdA* or *oatA* transposon mutants in macrophages, Δ*hly*Δ*pgdA*Δ*oatA* died rapidly (Fig 3.1D). Therefore, the individual mutants for which we observed increased IL-10 likely have minor increases in lysis that were not easily detected by CFU. However, in combination, the *pgdA* and *oatA* mutations synergized to yield a mutant that was extremely susceptible to lysis in cells and potently induced IL-10. Importantly, these data suggest that low-level lysis can be detected by TLRs and lead to significant changes in cytokine secretion.

The majority of mutants identified in our screen induced increased IL-10 secretion. Strikingly, while 50 mutants that induced increased IL-10 secretion were identified in our initial screen in Δ*hly*Δ*fla L. monocytogenes* (Supplemental Table 3.1), only 4 of those mutants induced significantly increased IL-10 secretion in a Δ*hly* background (Supplemental Table 3.2). It is possible that Δ*hly* may be slightly more sensitive to lysis compared to Δ*hly*Δ*fla*. Flagellar secretion systems require the activity of hydrolases or lytic transglycosylases to degrade peptidoglycan to allow insertion of flagellar components (187,188). Thus it is reasonable to suspect that the cell wall of Δ*hly* is more fragile than that of Δ*hly*Δ*fla* and lyses more inside vacuoles due to the activity of a flagella-associated peptidoglycan-degrading enzymes. An increase is the basal levels of lysis would increase the basal levels of IL-10 and potentially mask the effects of other mutations that slightly increase lysis. The results of this study suggest that any mutation that promotes bacterial lysis within a phagosome will lead to IL-10 induction.

In this study, we did not investigate whether a particular endosomal nucleic-acid sensing TLR is responsible for induction of IL-10, whether there is redundancy, or whether there is synergism. It is likely that all nucleic-acid sensing TLRs can be activated following bacterial

lysis. Bacterial lysis should lead to the release of bacterial contents including: mRNA that can activate TLR7/8, unmethylated CpG chromosomal DNA that can activate TLR9, and ribosomal RNA that can activate TLR13 (189). Although TLR3 could also recognize double-stranded RNA released upon bacterial lysis, it likely does not to contribute to IL-10 secretion because it was previously demonstrated that IL-10 secretion in response to vacuole-confined *L. monocytogenes* is dependent on the signaling adapter MyD88, and TLR3 uses the signaling adapter TRIF (130,189). It has been previously suggested that bacterial mRNA represents a signature of bacterial viability (190). Perhaps, then, simultaneous recognition of multiple types of nucleic acid by multiple TLRs is indicative of nonviable bacteria.

The observation that the immune system responds more robustly to bacteria that are alive compared to bacteria that are dead led to the idea that the immune system has ways of monitoring bacterial viability (190–192). Many signals have been proposed to be "PAMPs per vita" or "vita-PAMPs"—signatures of microbial viability. DNA and RNA have been proposed as possible "PAMP postmortem" (PAMP-PM). In line with the idea of recognition of postmortem PAMPs, the secretion of IL-10 following infection of mice with Δ*hly* could represent a strategy to prevent an unnecessary immune response to bacteria that are already dead, and thus do not pose a threat. To that end, nucleic acid-sensing TLRs are better suited for assessing bacterial viability than TLR2, because living bacteria do not normally release nucleic acids, especially chromosomal DNA, into the surrounding environment. Signaling through nucleic-acid sensing TLRs thus more accurately indicates that a bacterium is dead than sensing of lipoproteins, which can be detected whether a pathogen is alive or dead.

Induction of IL-10 secretion is an important factor to consider in the development of bacterial vaccine vectors. Both live and dead bacterial vaccine vectors have the potential to induce IL-10 secretion. The kinetics of IL-10 secretion may play an important role in determining whether a vaccine will be effective or not. In our study, we identified sensing of nucleic acids as the primary signal for IL-10 induction. For the development of future vaccine strains, strategies to minimize IL-10 induction and immune suppression should be considered. For example, mutations that modify the cell wall could be employed to reduce bacterial death in phagosomes. Also, as suggested above, simply deleting flagellin may decrease bacteriolysis. Mutations could also be made to alter lipoproteins and enhance TLR2 activation and proinflammatory cytokine. In combination, modifications that reduce IL-10 secretion and modulate proinflammatory cytokines downstream of TLR2 may yield a vaccine strain that has increased potency.

3.5 Materials and Methods

Strain Construction

In-frame deletion of genes was performed using allelic exchange as previously described (193). Δ*hly*Δ*fla* was generated by deleting *hly* in a Δ*flaA* strain (DP-L5986). Δ*hly*Δ*lgt* was generated by deleting *lgt* in a Δ*hly* strain (DP-L2161). Δ*hly*Δ*fla*Δ*lgt* was generated by deleting *lgt* in a Δ*hly*Δ*fla* strain. Δ*hly*Δ*pgdA*Δ*oatA* was generated by deleting *hly* in a Δ*pgdA*Δ*oatA* strain (DP-L5220). Δ*hly*Δ*fla*Δ*lgt* was generated by deleting *lgt* in a Δ*hly*Δ*fla* strain. The *lgt* complemented strains were generated by integrating a pPL2 vector encoding *lgt* under control of the pHyper promoter (pPL2t-*pHyper*-*lgt*) into the *L. monocytogenes* genome and selecting for tetracycline-resistant transconjugates (194).

Transposon Library Generation

A transposon library was generated in Δ*hly*Δ*fla* as previously described (195). Transposon mutations were transduced into Δ*hly* using U153 phage as previously described (196).

Bone Marrow-Derived Macrophage Culture

BMM growth media was prepared using high glucose DMEM (Thermo Fisher Scientific) with 20% Fetal Bovine Serum (Seradigm), 1% L-glutamine (Corning), 1% Sodium pyruvate (Corning), 14mM 2-Mercaptoethanol (Gibco), and 10% 3T3 cell supernatant (from M-CSF-producing 3T3 cells).

Intracellular Survival/Growth of *L. monocytogenes* in BMMs

3×10^6 BMMs were plated in 60 mm non-TC-treated Petri dishes with 14 12mm glass coverslips in each dish. Dishes were infected with 5×10^5 CFU (MOI=0.17) and intracellular growth curves were performed as described previously (96).

Quantification of IL-10 from BMMs – 96-well format

2.6×10^5 BMMs in 200 µL BMM growth media were seeded into wells of a 96-well plate. Bacteria were grown in 1 mL Brain-Heart Infusion Broth containing 200µg/mL streptomycin in 96-well deep well plates at 30°C. Prior to infection, bacteria were pelleted by centrifugation and resuspended in 1 mL PBS. Wells were infected with 8 µL of resuspended bacteria. Plates were infected in duplicate. 30 minutes post-infection, cells were washed with warm PBS, and BMM growth media with 50 µg/mL gentamicin was added. Supernatants were collected and frozen at -80°C until used for analysis. For quantification of IL-10, Mouse IL-10 DuoSet ELISA (R&D Systems) was performed according to manufacturer's instructions.

Quantification of IL-10 from BMMs – 24-well format

$6 \times 10^5 – 7 \times 10^5$ BMMs in 500 µL BMM growth media were seeded into wells of a 24-well plate. Bacteria were grown overnight at 30°C at a slant without shaking in 3 mL Brain-Heart Infusion Broth containing 200µg/mL streptomycin. Cultures were then pelleted by centrifugation, and resuspended in phosphate-buffered solution (PBS) to an optical density of 2.0. Wells were infected with 20µL of bacteria, approximately 8×10^7 CFU (MOI=120). Three wells were infected for each bacterial strain. 30 minutes post-infection, cells were washed with warm PBS, and BMM growth media with 50 µg/mL gentamicin was added. Supernatants were

collected and frozen at -80°C until used for analysis. For quantification of IL-10, Mouse IL-10 DuoSet ELISA (R&D Systems) was performed according to manufacturer's instructions.

Animal Use Ethics Statement

All animal work was done in strict accordance with university regulations. Protocols were reviewed and approved by the Animal Care and Use Committee at the University of California, Berkeley AUP-2016-05-8811.

Mice

C57BL/6J mice were purchased from Jackson Laboratories. TLR2$^{-/-}$, Unc93b1$^{3d/3d}$, and TLR2$^{-/-}$TLR5$^{-/-}$Unc93b1$^{3d/3d}$ were a gift from Greg Barton and were bred in our facility.

Quantification of cytokines from serum

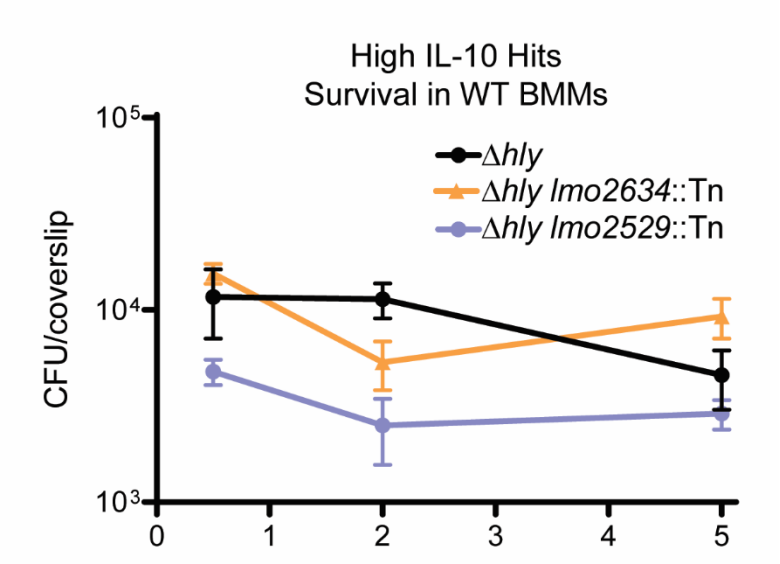
Eight-to-twelve week old female mice were injected via the tailvein with 10^8 CFU of Δ*hly* background strains or 10^5 CFU Δ*actA*. Bacteria were grown overnight at 30°C at a slant without shaking in Brain-Heart Infusion Broth containing 200μg/mL streptomycin. Bacteria were then backdiluted 1:20 and grown at 37°C shaking for about two hours until they reached an optical density of 0.5. Cultures were pelleted and resuspended in PBS to the appropriate concentration, such that mice were infected with 200μL. Four and twenty-four hours post infection, blood was collected from the submandibular vein into Microtainer tubes with serum separator additive (BD). Blood was left to rest for 30 minutes before tubes were centrifuged and serum was collected. Collected serum was stored at -20°C until analysis. Analysis of cytokines from serum was performed using Mouse Inflammation Cytokine Bead Arrays (BD, Cat. 552364). Data was analyzed using FlowJo.

Vaccination and Immune Suppression

Eight-to-twelve week old female mice were vaccinated intravenously via the tailvein with 10^8 CFU of Δ*hly* background strains or 10^5 CFU Δ*actA*. Bacteria were grown overnight at 30°C at a slant without shaking in Brain-Heart Infusion Broth containing 200μg/mL streptomycin. Bacteria were then backdiluted 1:20 and grown at 37°C shaking for about two hours until they reached an optical density of 0.5. Cultures were pelleted and resuspended in PBS to the appropriate concentration, such that mice were vaccinated with 200 μL. Eight weeks post-vaccination, mice were challenged with 5 x 10^4 CFU WT *L. monocytogenes.* Three days post-challenge, mice were euthanized and CFU in the spleens and livers were enumerated.

Statistical Analysis

Data were analyzed using GraphPad Prism 8. For mouse CFU experiments, data was log-transformed prior to performing statistical analysis. * indicates P <0.05; ** indicates P <0.01, *** indicates P <0.001, **** indicates P <0.0001; ns indicates no statistical significance.



Supplemental Figure 3.1. Survival of transposon mutants in WT BMMs.

Survival of transposon mutants was quantified in BMMs. Mean and SEM are shown. Data is pooled from three coverslips per experiment for three independent experiments.

Supplemental Table 3.1. IL-10 secretion of transposon mutants in Δ*hly*Δ*fla* background compared to Δ*hly*Δ*fla*.

Lmo	**Gene Annotation**	**Mean IL-10 (% Δ*hly*Δ*fla* IL-10*)***	**Standard Deviation**
0095	promoter region of lmo0095	162	14
0286	pyridoxal phosphate-dependent aminotransferase	202	25
0331	LPXTG-motif cell wall anchor domain-containing protein	137	6
0333	inlI, LPXTG-motif cell wall anchor domain-containing protein	165	28
0367	deferrochelatase/peroxidase EfeB	142	7
0371	GntR family transcriptional regulator	77	8
0415	Peptidoglycan/xylan/chitin deacetylase, PgdA/CDA1 family	172	8
0497	Glycosyl transferase family 2	151	24
0524	sulfate transporter	134	1
0580	Phospholipase/Carboxylesterase	184	7
0635	HAD family hydrolase	153	17
Lmo0671-lmo0672	Uncharacterized membrane protein YhaH, DUF805 family	153	12
0709	Hypothetical protein	68	4
0769	alpha-1,6-mannanase	226	37
0785	sigma54-associated activator ManR	131	2
0842	putative peptidoglycan bound protein (LPXTG motif)	171	28
0848	amino acid ABC transporter ATP-binding protein	171	19
0954	promoter region of lmo0954	73	6
1080	teichoic acid biosynthesis protein GgaB	64	13
1131	ABC transporter ATP-binding protein	245	14
1140	hypothetical protein	161	1
1241	hypothetical protein	158	10
1291	(oatA) acetyltransferase to YrhL	158	15
1293	glycerol-3-phosphate dehydrogenase, glpD	77	8
1296	GTPase HflX	142	16
1366	23S rRNA (cytidine1920-2'-O)/16S rRNA (cytidine1409-2'-O)-methyltransferase	135	2
1395	protein RodZ, contains Xre-like HTH and DUF4115 domains	126	4
1429	energy-coupled thiamine transporter ThiT	125	9

1499	endolytic transglycosylase MltG	153	26
1652	multidrug ABC transporter permease/ATP-binding protein	*130*	7
1695	mprF	145	17
1742	adeC	154	25
1775	promoter region, purE, phosphoribosylaminoimidazole carboxylase catalytic subunit	144	2
1799	putative peptidoglycan bound protein (LPXTG motif)	252	11
1835	pyrAB	213	29
1843	RluA family pseudouridine synthase	153	19
1877	formyl-tetrahydrofolate synthetase	151	11
1956	Fur family transcriptional regulator, ferric uptake regulator	77	3
2027	putative cell surface protein, similar to internalin proteins	198	10
2079	hypothetical protein	78	8
2128	transcriptional regulator, LacI family	178	11
2229	penicillin-binding protein 2A	141	10
2287	putative tape-measure [Bacteriophage A118]	183	5
2389	NADH dehydrogenase	146	18
2482	*(lgt)* prolipoprotein diacylglyceryl transferase	9	1
2529	ATP synthase F0F1 subunit beta	159	22
2530	atpG	146	27
2531	ATP synthase F0F1 subunit alpha	16	2
2581	promoter region of ABC transporter permease	154	13
2634	energy-coupling factor transporter transmembrane protein EcfT	164	23
2634	energy-coupling factor transporter transmembrane protein EcfT	135	8
2641	heptaprenyl diphosphate synthase	163	19
2720	acyl--CoA ligase	161	29
2757	DNA helicase RecQ	152	5
2760	ABC transporter ATP-binding protein	174	19
2816	Sugar phosphate permease	132	11
2835	xylose isomerase	172	20
2835	xylose isomerase	165	1
2854	Membrane protein insertase YidC 2	156	30

Supplemental Table 3.2. IL-10 secretion of transposon mutants in Δ*hly* background compared to Δ*hly*.

Lmo	**WT$^{\alpha}$**	**Sign.$^{\beta}$**	**TLR2$^{\alpha}$**	**Sign.$^{\beta}$**	**Unc93b1$^{3d/3d\ \alpha}$**	**Sign.$^{\beta}$**	**TLR2$^{-/-}$TLR5$^{-/-}$ Unc93b1$^{3d/3d\ \alpha}$**
0005	88	ns	114	ns	125	ns	µ
0286	118	ns	96	ns	105	ns	µ
0331	97	ns	131	ns	128	ns	µ
0333	119	ns	111	ns	130	ns	µ
0367	116	ns	134	ns	142	ns	µ
0371	101	ns	177	**	130	*	µ
0415	177	****	493	****	174	****	µ
0497	89	ns	108	ns	116	ns	µ
0524	108	ns	136	ns	155	*	µ
0580	96	ns	131	ns	115	ns	µ
0635	127	ns	139	ns	142	ns	µ
Lmo0671 -lmo0672	104	ns	128	ns	117	ns	µ
0709	41	****	64	ns	77	ns	µ
0769	133	ns	185	ns	144	**	µ
0785	120	ns	118	ns	128	ns	µ
0842	170	ns	153	ns	175	****	µ
0848	85	ns	90	ns	112	ns	µ
0954	90	ns	100	ns	113	ns	µ
1131	114	ns	148	ns	152	***	µ
1140	124	ns	106	ns	124	ns	µ
1241	109	ns	135	ns	121	ns	µ
1291	139	**	327	****	184	****	µ
1293	122	ns	137	ns	109	ns	µ
1296	138	ns	121	ns	143	*	µ
1366	102	ns	128	ns	145	*	µ
1395	91	ns	113	ns	146	ns	µ
1429	124	ns	184	**	127	ns	µ
1499	114	ns	137	ns	115	ns	µ
1652	97	ns	148	*	148	ns	µ
1695	99	ns	86	ns	179	***	µ
1742	126	ns	152	ns	176	***	µ
1775	107	ns	200	***	136	ns	µ
1799	108	ns	134	ns	137	ns	µ
1835	135	ns	103	ns	165	****	µ
1843	127	ns	154	ns	124	ns	µ
1877	125	ns	133	ns	123	ns	µ
1956	97	ns	207	****	129	ns	µ
2027	118	ns	147	*	114	ns	µ
2079	73	ns	139	ns	131	ns	µ

2128	119	ns	133	ns	131	*	µ
2229	122	ns	133	ns	148	****	µ
2287	114	ns	108	ns	132	ns	µ
2389	88	ns	162	ns	112	ns	µ
2482	27	****	162	**	4	****	µ
2529	158	*	221	****	162	**	µ
2530	209	ns	251	****	118	ns	µ
2531	113	ns	212	***	106	ns	µ
2581	96	ns	177	*	160	***	µ
2634	140	*	145	*	154	**	µ
2634	185	ns	342	*	158	**	µ
2641	95	ns	194	***	135	ns	µ
2720	89	ns	105	ns	113	ns	µ
2757	108	ns	105	ns	156	ns	µ
2760	95	ns	100	ns	124	ns	µ
2816	102	ns	170	ns	116	ns	µ
2835	90	ns	151	ns	109	ns	µ
2854	136	ns	134	ns	167	ns	µ

Values reported are percent IL-10 normalized to 100% Δ*hly* IL-10. [α] BMM background. [µ]IL-10 secretion <80pg/mL. [β]Δ*hly* transposon mutant IL-10 values were compared to Δ*hly* IL-10 values using Dunnett's multiple comparisons test. * indicates these values are statistically significant, with a p-value <0.05; ** indicates a p-value <0.01; *** indicates a p-value <0.001; **** indicates a p-value <0.0001; ns indicates no statistical significance.

Supplemental Table 3.3. List of Strains.

Strain name	Strain background	Lmo of Gene with Transposon Insertion	Integrated Plasmid	Strain Number	Reference
Δ*hly*	Δ*hly*	-	-	DP-L2161	(2)
Δ*flaA*	Δ*flaA*			DP-L5986	This study
hly knockout plasmid	*E. coli*	-	-	DP-L2154	(2)
Δ*hly*Δ*fla*	Δ*hly*Δ*fla*	-	-	DP-L7001	This study
lgt knockout plasmid	*E. coli*	-	-	DP-E7002	This study
Δ*hly*Δ*lgt*	Δ*hly*Δ*lgt*	-	-	DP-L7003	This study
Δ*pgdA*Δ*oatA*	Δ*pgdA*Δ*oatA*	-	-	DP-L5220	(171)
Δ*hly*Δ*pgdA*Δ*oatA*	Δ*hly*Δ*pgdA*Δ*oatA*	-	-	DP-L7004	This study
Δ*hly*Δ*fla*Δ*lgt*	Δ*hly*Δ*fla*Δ*lgt*	-	-	DP-L7005	This study
pPL2t-*pHyper-lgt*	*E. coli*	-	-	DP-E7006	This study
Δ*hly*Δ*lgt* + *lgt*	Δ*hly*Δ*lgt*	-	pPL2t-*pHyper-lgt*	DP-L7007	This study
Δ*hly*Δ*fla*Δ*lgt* + *lgt*	Δ*hly*Δ*fla*Δ*lgt*	-	pPL2t-*pHyper-lgt*	DP-L7008	This study
ΔactA	ΔactA	-	-	DP-L4029	(194)
	Δ*hly*Δ*fla*	*Lmo0095*		DP-L7009	This study
	Δ*hly*Δ*fla*	*Lmo0286*		DP-L7010	This study
	Δ*hly*Δ*fla*	*Lmo0331*		DP-L7011	This study
	Δ*hly*Δ*fla*	*Lmo0333*		DP-L7012	This study
	Δ*hly*Δ*fla*	*Lmo0367*		DP-L7013	This study
	Δ*hly*Δ*fla*	*Lmo0371*		DP-L7014	This study
	Δ*hly*Δ*fla*	*Lmo0*415		DP-L7015	This study
	Δ*hly*Δ*fla*	*Lmo0*497		DP-L7016	This study
	Δ*hly*Δ*fla*	*Lmo0524*		DP-L7017	This study
	Δ*hly*Δ*fla*	*Lmo0*580		DP-L7018	This study
	Δ*hly*Δ*fla*	*Lmo0635*		DP-L7019	This study
	Δ*hly*Δ*fla*	*Intergenic; Lmo0*671-*Lmo0*672		DP-L7020	This study
	Δ*hly*Δ*fla*	*Lmo0*709		DP-L7021	This study
	Δ*hly*Δ*fla*	*Lmo0769*		DP-L7022	This study
	Δ*hly*Δ*fla*	*Lmo0785*		DP-L7023	This study
	Δ*hly*Δ*fla*	*Lmo0842*		DP-L7024	This study
	Δ*hly*Δ*fla*	*Lmo0848*		DP-L7025	This study
	Δ*hly*Δ*fla*	*Lmo0954*		DP-L7026	This study

	Δ*hly*Δ*fla*	*Lmo1080*		DP-L7027	This study
	Δ*hly*Δ*fla*	*Lmo1131*		DP-L7028	This study
	Δ*hly*Δ*fla*	*Lmo1140*		DP-L7029	This study
	Δ*hly*Δ*fla*	*Lmo1241*		DP-L7030	This study
	Δ*hly*Δ*fla*	*Lmo1291*		DP-L7031	This study
	Δ*hly*Δ*fla*	*Lmo1293*		DP-L7032	This study
	Δ*hly*Δ*fla*	*Lmo1296*		DP-L7033	This study
	Δ*hly*Δ*fla*	*Lmo1366*		DP-L7034	This study
	Δ*hly*Δ*fla*	*Lmo1395*		DP-L7035	This study
	Δ*hly*Δ*fla*	*Lmo1429*		DP-L7036	This study
	Δ*hly*Δ*fla*	*Lmo1499*		DP-L7037	This study
	Δ*hly*Δ*fla*	*Lmo1652*		DP-L7038	This study
	Δ*hly*Δ*fla*	*Lmo1695*		DP-L7039	This study
	Δ*hly*Δ*fla*	*Lmo1742*		DP-L7040	This study
	Δ*hly*Δ*fla*	*Lmo1775*		DP-L7041	This study
	Δ*hly*Δ*fla*	*Lmo1799*		DP-L7042	This study
	Δ*hly*Δ*fla*	*Lmo1835*		DP-L7043	This study
	Δ*hly*Δ*fla*	*Lmo1843*		DP-L7044	This study
	Δ*hly*Δ*fla*	*Lmo1877*		DP-L7045	This study
	Δ*hly*Δ*fla*	*Lmo1956*		DP-L7046	This study
	Δ*hly*Δ*fla*	*Lmo2027*		DP-L7047	This study
	Δ*hly*Δ*fla*	*Lmo2079*		DP-L7048	This study
	Δ*hly*Δ*fla*	*Lmo2128*		DP-L7049	This study
	Δ*hly*Δ*fla*	*Lmo2229*		DP-L7050	This study
	Δ*hly*Δ*fla*	*Lmo2287*		DP-L7051	This study
	Δ*hly*Δ*fla*	*Lmo2389*		DP-L7052	This study
	Δ*hly*Δ*fla*	*Lmo2482*		DP-L7053	This study
	Δ*hly*Δ*fla*	*Lmo2529*		DP-L7054	This study
	Δ*hly*Δ*fla*	*Lmo2530*		DP-L7055	This study
	Δ*hly*Δ*fla*	*Lmo2531*		DP-L7056	This study
	Δ*hly*Δ*fla*	*Lmo2581*		DP-L7057	This study
	Δ*hly*Δ*fla*	*Lmo2634*		DP-L7058	This study
	Δ*hly*Δ*fla*	*Lmo2634*		DP-L7059	This study
	Δ*hly*Δ*fla*	*Lmo2641*		DP-L7060	This study
	Δ*hly*Δ*fla*	*Lmo2720*		DP-L7061	This study
	Δ*hly*Δ*fla*	*Lmo2757*		DP-L7062	This study
	Δ*hly*Δ*fla*	*Lmo2760*		DP-L7063	This study
	Δ*hly*Δ*fla*	*Lmo2816*		DP-L7064	This study
	Δ*hly*Δ*fla*	*Lmo2835*		DP-L7065	This study

	Δ*hly*Δ*fla*	*Lmo2835*		DP-L7066	This study
	Δ*hly*Δ*fla*	*Lmo2854*		DP-L7067	This study
	Δ*hly*	*Lmo0095*		DP-L7068	This study
	Δ*hly*	*Lmo0286*		DP-L7069	This study
	Δ*hly*	*Lmo0331*		DP-L7070	This study
	Δ*hly*	*Lmo0333*		DP-L7071	This study
	Δ*hly*	*Lmo0367*		DP-L7072	This study
	Δ*hly*	*Lmo0371*		DP-L7073	This study
Δ*hly pgdA::*Tn	Δ*hly*	*Lmo0*415		DP-L7074	This study
	Δ*hly*	*Lmo0*497		DP-L7075	This study
	Δ*hly*	*Lmo0524*		DP-L7076	This study
	Δ*hly*	*Lmo0*580		DP-L7077	This study
	Δ*hly*	*Lmo0635*		DP-L7078	This study
	Δ*hly*	*Intergenic; Lmo0*671-*Lmo0*672		DP-L7079	This study
Δ*hly lmo0709::*Tn	Δ*hly*	*Lmo0*709		DP-L7080	This study
	Δ*hly*	*Lmo0769*		DP-L7081	This study
	Δ*hly*	*Lmo0785*		DP-L7082	This study
	Δ*hly*	*Lmo0842*		DP-L7083	This study
	Δ*hly*	*Lmo0848*		DP-L7084	This study
	Δ*hly*	*Lmo0954*		DP-L7085	This study
	Δ*hly*	*Lmo1131*		DP-L7086	This study
	Δ*hly*	*Lmo1140*		DP-L7087	This study
	Δ*hly*	*Lmo1241*		DP-L7088	This study
Δ*hly oatA::*Tn	Δ*hly*	*Lmo1291*		DP-L7089	This study
	Δ*hly*	*Lmo1293*		DP-L7090	This study
	Δ*hly*	*Lmo1296*		DP-L7091	This study
	Δ*hly*	*Lmo1366*		DP-L7092	This study
	Δ*hly*	*Lmo1395*		DP-L7093	This study
	Δ*hly*	*Lmo1429*		DP-L7094	This study
	Δ*hly*	*Lmo1499*		DP-L7095	This study
	Δ*hly*	*Lmo1652*		DP-L7096	This study
	Δ*hly*	*Lmo1695*		DP-L7097	This study
	Δ*hly*	*Lmo1742*		DP-L7098	This study
	Δ*hly*	*Lmo1775*		DP-L7099	This study
	Δ*hly*	*Lmo1799*		DP-L7100	This study
	Δ*hly*	*Lmo1835*		DP-L7101	This study
	Δ*hly*	*Lmo1843*		DP-L7102	This study

	Δ*hly*	*Lmo1877*		DP-L7103	This study
	Δ*hly*	*Lmo1956*		DP-L7104	This study
	Δ*hly*	*Lmo2027*		DP-L7105	This study
	Δ*hly*	*Lmo2079*		DP-L7106	This study
	Δ*hly*	*Lmo2128*		DP-L7107	This study
	Δ*hly*	*Lmo2229*		DP-L7108	This study
	Δ*hly*	*Lmo2287*		DP-L7109	This study
	Δ*hly*	*Lmo2389*		DP-L7110	This study
Δ*hly lgt::*Tn	Δ*hly*	*Lmo2482*		DP-L7111	This study
Δ*hly lmo2529::*Tn	Δ*hly*	*Lmo2529*		DP-L7112	This study
	Δ*hly*	*Lmo2530*		DP-L7113	This study
	Δ*hly*	*Lmo2531*		DP-L7114	This study
	Δ*hly*	*Lmo2581*		DP-L7115	This study
Δ*hly lmo2634::*Tn	Δ*hly*	*Lmo2634*		DP-L7116	This study
	Δ*hly*	*Lmo2634*		DP-L7117	This study
	Δ*hly*	*Lmo2641*		DP-L7118	This study
	Δ*hly*	*Lmo2720*		DP-L7119	This study
	Δ*hly*	*Lmo2757*		DP-L7120	This study
	Δ*hly*	*Lmo2760*		DP-L7121	This study
	Δ*hly*	*Lmo2816*		DP-L7122	This study
	Δ*hly*	*Lmo2835*		DP-L7123	This study
	Δ*hly*	*Lmo2835*		DP-L7124	This study
	Δ*hly*	*Lmo2854*		DP-L7125	This study

Chapter 4: Concluding Thoughts and Unanswered Questions

4.1 Conclusion and outstanding questions

In this work, we set out to answer a simple question: 'does LLO in the cytosol have any effects on the cell or pathogenesis?' Despite the simplicity of that question, it has been technically challenging to come up with any satisfying answers. We designed a tool *hly*^{fl} to address that question, and we've determined that the answer is yes. LLO in the cytosol can be cytotoxic. Like with all findings in science, this result opened the door to so many other questions. What type of cytotoxicity does LLO cause? How does LLO-dependent cytotoxicity affect pathogenesis? In our search for answers we found even more questions. What type of cell death does *L. monocytogenes* cause, in general? Does *L. monocytogenes* have a mechanism for inhibiting cell death thereby protecting its intracellular niche? Our findings suggest that it might. Then there are the even harder questions, the 'why' questions. Why does *L. monocytogenes* continue to secrete LLO in the cytosol when it will eventually lead to death of the host cell? Is it because LLO is needed for immediate escape from secondary vacuoles? Does LLO in the cytosol initiate a host response that contributes to pathogenesis? We hope that *hly*^{fl} will allow others to continue asking and answering questions about the role of LLO in pathogenesis.

To complement the work we've done on what happens during infections when LLO *is* present, we then investigated what happens when it *isn't.* For thirty years it has been known that LLO is required for escape from the vacuole and therefore is required for pathogenesis. End of story. Not so fast – we've also known for a long time that LLO is not required for vacuolar escape in HeLa cells. At times this has been written off as an oddity of HeLa cells and at others it has been used as a tool to circumvent the requirement for LLO. What hasn't been asked is: is there more to this story? How does *L. monocytogenes* escape from vacuoles in HeLa cells without perforating them with LLO? Are there mammalian host cells *in vivo* that behave similarly? Does it have anything to do with the cancerous properties of HeLa cells? The last two questions are increasingly relevant because of the use of *L. monocytogenes* as an anti-cancer therapy. If there is a tumor type that permits *L. monocytogenes* invasion into the cytosol without LLO, perhaps a Δ*hly* strain injected directly into the tumor would be effective and safe.

In this study, we investigated how the host recognizes and responds to Δ*hly L. monocytogenes* infection. When we started this study, we hoped that if we could identify the *L. monocytogenes* component that induces IL-10 we could generate a Δ*hly L. monocytogenes* mutant strain that would not induce IL-10 and would therefore be an ideal vaccine delivery strain. We found that recognition of nucleic acids by endosomal TLRs and recognition of lipoproteins by TLR2, likely following bacteriolysis in the phagosomal compartment, leads to production of IL-10 by the host. We identified more than 50 mutations that likely increase *L. monocytogenes* susceptibility to bacteriolysis. Next, we need to identify mutations or strategies for manipulating bacteria to lyse less.

Deleting *hly* remains the most effective way to attenuate *L. monocytogenes in vivo* without affecting growth outside of cells. It still has potential as a vaccine stain if we can find a way to reduce bacteriolysis. On the other hand, that is perhaps an insurmountable challenge. Why not use Δ*hly L. monocytogenes* as it is? We, and others, have demonstrated that Δ*hly L. monocytogenes* induces potent immunosuppression. Can we take advantage of that to treat inflammatory and autoimmune disorders? This possibility has not been adequately explored, and there is so much potential.

4.2 The Future

All of the work I've done over the past few years has been guided by the hope of finding a strain of *L. monocytogenes* that is safer and/or elicits a stronger adaptive immune response than Δ*actA*. It seems impossible that over the course of 20 years of *Listeria* research, no one has found a strain that works better, yet that continues to be the case. I think that a better vaccine strain exists, and that we haven't found it yet suggests there is still much for us to learn. With *L. monocytogenes* as a tool, there is a future full of advancements in the fields of cell biology, immunology, and pathogenesis.

Chapter 5: References

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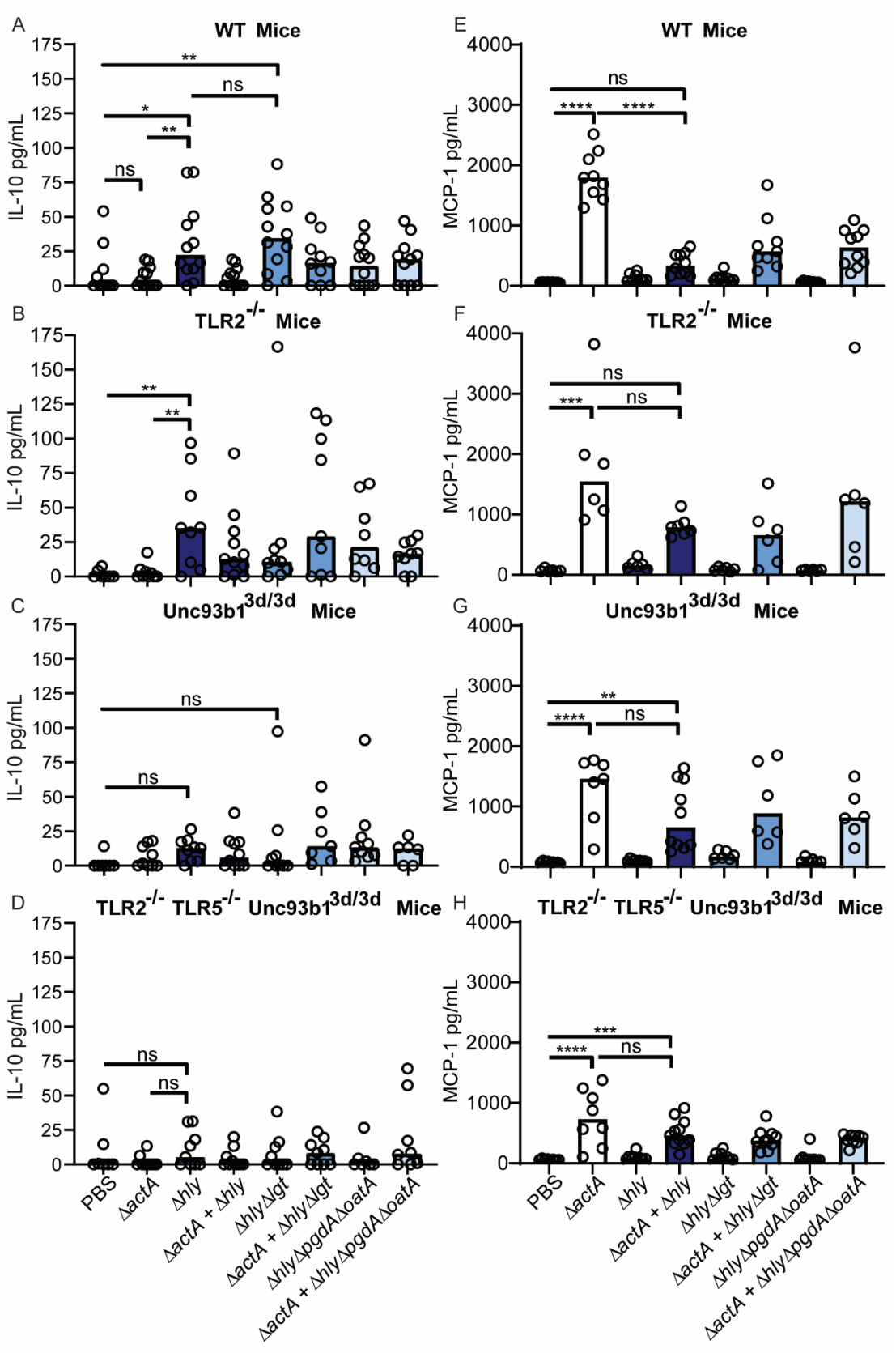
Chapter 6: Appendix


A
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IL-10 pg/mL
B
TLR2-/- Mice
C
Unc93b13d/3d Mice
D
TLR2-/- TLR5-/- Unc93b13d/3d Mice
E
WT Mice
MCP-1 pg/mL
F
TLR2-/- Mice
G
Unc93b13d/3d Mice
H
TLR2-/- TLR5-/- Unc93b13d/3d Mice
PBS
ΔactA
Δhly
ΔactA + Δhly
ΔhlyΔlgt
ΔactA + ΔhlyΔlgt
ΔhlyΔpgdAΔoatA
ΔactA + ΔhlyΔpgdAΔoatA

Figure 5.1. *L. monocytogenes*-induced IL-10 and MCP-1 secretion in mice.

Mice were infected with 10^8 CFU of Δ*hly*, 10^5 CFU of Δ*actA*, or a combination of 10^8 CFU of Δ*hly* and 10^5 CFU of Δ*actA*. WT C57BL/6J mice (A and E), $TLR2^{-/-}$ (B and F), $Unc93b1^{3d/3d}$ (C and G), and $TLR2^{-/-}TLR5^{-/-}Unc93b1^{3d/3d}$ (D and H) were infected. Serum measurements of IL-10 four hours post-infection (A-D) and MCP-1 twenty-four hours post-infection (E-H). Data is pooled from two to four independent experiments. Bar represents the median. Data analyzed using Holm-Sidak's Multiple Comparisons test.

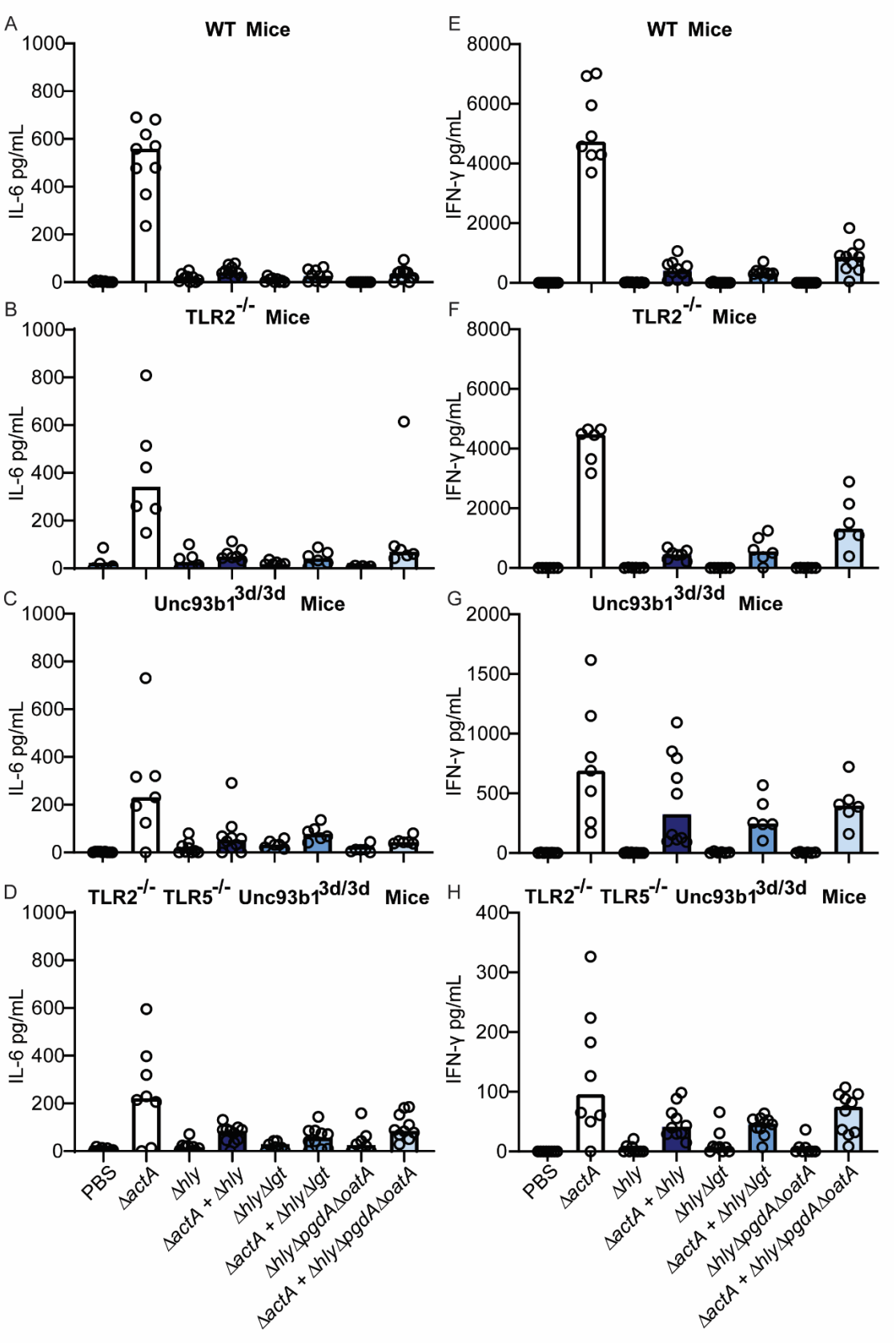
A
WT Mice
IL-6 pg/mL
1000
800
600
400
200
0
E
WT Mice
IFN-γ pg/mL
8000
6000
4000
2000
0
B
TLR2-/- Mice
IL-6 pg/mL
F
TLR2-/- Mice
IFN-γ pg/mL
C
Unc93b13d/3d Mice
IL-6 pg/mL
G
Unc93b13d/3d Mice
IFN-γ pg/mL
2000
1500
1000
500
0
D
TLR2-/- TLR5-/- Unc93b13d/3d Mice
IL-6 pg/mL
H
TLR2-/- TLR5-/- Unc93b13d/3d Mice
IFN-γ pg/mL
400
300
200
100
0
PBS
ΔactA
Δhly
ΔactA + Δhly
ΔhlyΔlgt
ΔactA + ΔhlyΔlgt
ΔhlyΔpgdAΔoatA
ΔactA + ΔhlyΔpgdAΔoatA

Figure 5.2. *L. monocytogenes*-induced IL-6 and IFN-γ secretion in mice.

Mice were infected with 10^8 CFU of Δ*hly*, 10^5 CFU of Δ*actA*, or a combination of 10^8 CFU of Δ*hly* and 10^5 CFU of Δ*actA*. WT C57BL/6J mice (A and E), $TLR2^{-/-}$ (B and F), $Unc93b1^{3d/3d}$ (C and G), and $TLR2^{-/-}TLR5^{-/-}Unc93b1^{3d/3d}$ (D and H) were infected. Serum measurements of IL-6 (A-D) and IFN-γ four hours post-infection (E-H). Data is pooled from two to four independent experiments. Bar represents the median.

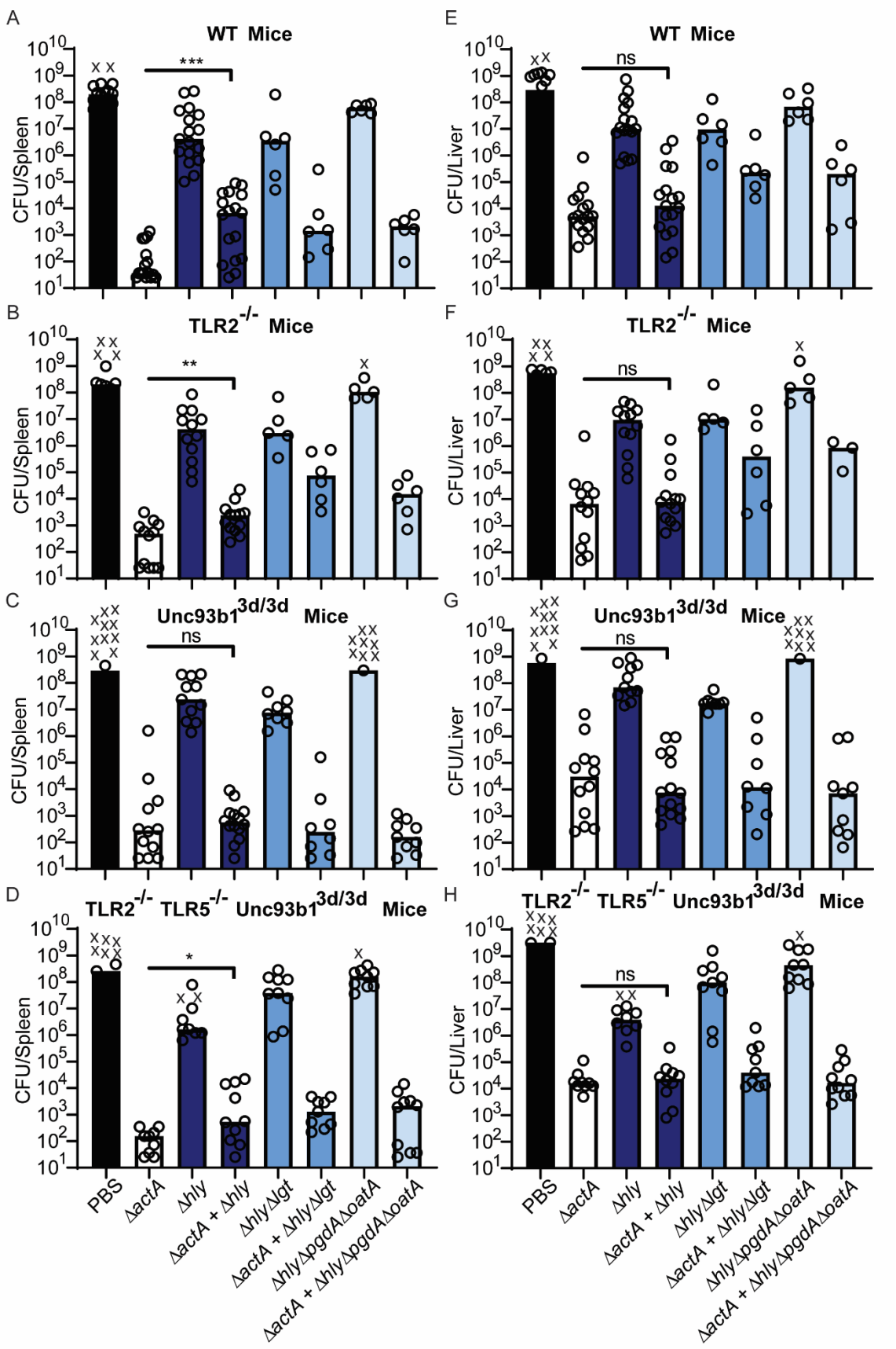
A
WT Mice
CFU/Spleen
***
B
TLR2-/- Mice
CFU/Spleen
**
C
Unc93b13d/3d Mice
CFU/Spleen
ns
D
TLR2-/- TLR5-/- Unc93b13d/3d Mice
CFU/Spleen
*
E
WT Mice
CFU/Liver
ns
F
TLR2-/- Mice
CFU/Liver
ns
G
Unc93b13d/3d Mice
CFU/Liver
ns
H
TLR2-/- TLR5-/- Unc93b13d/3d Mice
CFU/Liver
ns
PBS
ΔactA
Δhly
ΔactA + Δhly
ΔhlyΔlgt
ΔactA + ΔhlyΔlgt
ΔhlyΔpgdAΔoatA
ΔactA + ΔhlyΔpgdAΔoatA

Figure 5.3. Immune suppression is primarily mediated by endosomal TLR signaling.

Mice were infected with 10^8 CFU of Δ*hly* background strains, 10^5 CFU of Δ*actA*, or a combination of 10^8 CFU of Δ*hly* background strains and 10^5 CFU of Δ*actA*. WT C57BL/6J mice (A), TLR2$^{-/-}$ (B), Unc93b1$^{3d/3d}$ (C), and TLR2$^{-/-}$TLR5$^{-/-}$Unc93b1$^{3d/3d}$ (D) were infected. Four-to-eight weeks post-vaccination, mice were challenged with $5x10^5$ WT *L. monocytogenes.* CFU from the spleen were enumerated three days post-challenge. Data is pooled from two to five independent experiments. Bar represents the median. Data analyzed using an unpaired t test.